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UNIVERSITY OF ALBERTA

BLOCKADE OF VOLTAGE-DEPENDENT SLOW CALCIUM CHANNELS IN FROG SKELETAL MUSCLE BY NON-PEPTIDE OPIOID DRUGS

BY

TUSHAR G. KOKATE (C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL 1990

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To my uncle

Dr. C. K. Kokate

for his constant inspiration

ABSTRACT

Methadone and several other non-peptide opioid agonists inhibited K^+ contractures and experimentally produced Ca^{++}-dependent slow action potentials (APs) in frog skeletal muscle by blocking voltage-dependent slow calcium channels. This effect was nonstereospecific and resistant to antagonism by naloxone. Although many non-peptide opioids were effective in blocking voltage-dependent slow calcium channels, various opioid peptides produced little or no effect on these channels.

Non-peptide opioids, in concentrations that blocked K^+ contractures did not modify K^+-induced membrane depolarization. Although non-peptide opioids completely blocked K^+ contractures, they did not produce any effect on caffeine-induced contractures, and in effective concentrations only potentiated twitches. These results indicated that non-peptide opioids did not deplete intracellular calcium stores or inhibit the release of calcium from intracellular sarcoplasmic reticulum stores. Elevating the calcium concentration in the bathing solution reversed non-peptide opioid-induced block of K^+ contractures.

Inhibition of K^+ contractures by non-peptide opioids was dependent on the frequency of K^+ contracture tests. Thus, block was increased and accelerated by increasing the frequency of K^+ contracture tests. Partial depolarization (-60 mV) of the skeletal muscle membrane in a chloride-free medium accelerated and also potentiated the non-peptide opioid-induced inhibition of K^+ contractures. These results suggested that binding of non-peptide opioids to their receptors is voltage-

dependent, with depolarization favoring the binding and accelerating the block. Two explanations were proposed for the frequency -and voltage-dependent effects of non-peptide opioids on slow calcium channels: firstly that the non-peptide opioids shift the steady-state inactivation curve for the slow calcium channels towards more negative potentials, secondly that the calcium channels are continuously active in partially depolarized muscle, thus allowing the non-peptide opioids to reach their receptors in the channels more effectively than in normally polarized cells.

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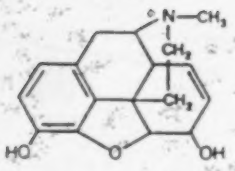
1. INTRODUCTION

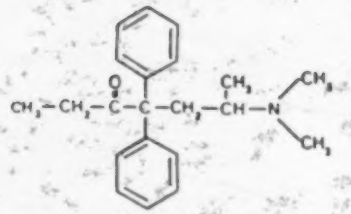
1.1 OPIOID HISTORY

Opium is an extract of the dried juice of the poppy plant, *Papaver somniferum (Papaveraceae)*, which has been used for social and medical purposes for thousands of years as an agent to produce euphoria, analgesia and sleep. The milky juice is taken from the seed capsules just prior to ripening and then dried and powdered. The word *opium* itself is derived from the Greek name for juice. By the middle of the sixteenth century, the uses of opium which are still valid were fairly well understood. In 1680, Syndenham wrote (*c.f.*[1]): "Among the remedies which it has pleased Almighty God to give to man to relieve his sufferings, none is so universal and so efficacious as opium."

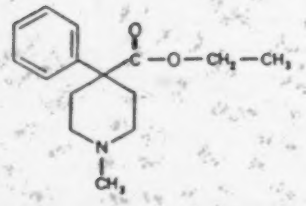
The modern basis of opioid pharmacology was established by Sertürner, who described the isolation of a pure active alkaloid from opium in 1803 (*c.f.*[1]). He proposed the name "morphine" for the compound after Morpheus, the Greek god of dreams. It was soon discovered that morphine was the major alkaloid responsible for most of the pharmacological and medicinal effects of opium. Since then, the drug has opened a whole vista of research for innumerable scientists. The problem of addiction to opioids stimulated a search for potent analgesics that would be free of the potential to produce addiction. Today, effective substitutes for morphine are available, but the ideal analgesic has yet to be developed (Fig. 1).



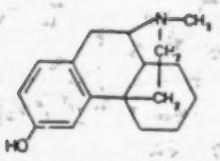
Morphine



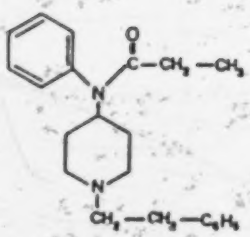
Methadone



Meperidine (Demerol)



Levorphanol (Levo-Dromoran)



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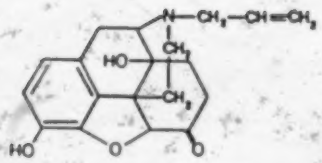

Naloxone (Narcan)

FIG. 1 The chemical structures of some of the opioid analgesics and naloxone, an opioid antagonist.

1.2 OPIOID RECEPTORS

The existence of a specific opioid receptor was first suggested by early behavioral and clinical studies in which morphine-like analgesics were shown to exhibit a high degree of structural and steric specificity [2,3].

The initial experiments aimed at directly demonstrating opioid receptors, either by studying the binding of opioids to nervous tissues *in vitro* or by examining the brain distribution of opioids after injection into animals *in vivo* were unsuccessful [4-7]. The main drawback of these studies was the inability to demonstrate a high-affinity binding component in brain tissue, which was taken as a necessary criterion of receptor binding. A major reason for this failing was that there was a large amount of non-specific binding in the earlier experiments which overshadowed the small fraction of specific binding to the opioid receptor sites. This problem was not totally overcome by Goldstein *et al.* [8], who nevertheless demonstrated a specific binding component in mouse brain that accounted for 2% of the total binding.

Subsequently, the availability of tritiated opioids with high specific radioactivity enabled the use of extremely low concentrations of opioids in binding studies. With these highly labelled compounds, several groups demonstrated independently a high-affinity binding of opioids to rat brain tissue [9-11]. This binding appeared to reflect

the interaction of the compounds with pharmacologically-relevant receptor sites. A great deal of work was later devoted to the characterization of the stereospecific binding sites and to the accumulation of evidence to identify these as the postulated opioid receptors (see [12]).

Several workers have found an excellent correlation between the binding affinities and the pharmacological activity of opioids [13-15]. However, caution must be exercised when interpreting specific binding of drugs as "receptor" binding. Since binding assays provide information only about the interaction of drugs with the recognition site of the receptor, it is therefore, necessary to relate binding data with the pharmacological response of the drug.

Isolated tissue preparations have found increasing use in the field of opioid research for numerous reasons. A primary advantage of investigating opioid receptor mechanisms in an isolated system is that the analysis of drug effects is less likely to be complicated by secondary factors such as distribution, metabolism and excretion. Thus, *in vitro* bioassay systems have been used extensively for rigorous characterization of opioid receptor interactions [16-20]. Peripheral tissue preparations in particular have proven to be invaluable model for analysis of opioid effects *in vitro*. To date, the most commonly used tissue preparations have been the guinea-pig ileum and mouse vas deferens [16-18]. One reason for this is that there

is a high degree of correlation between opioid agonist potency in these peripheral tissue preparations and the clinical analgesic activity of opioid drugs[18].

1.2.1 **Discovery of endogenous opioid ligands**

In 1971, Collier [21] explicitly postulated the existence of an "endogenous neurohumoral factor" interacting with the opioid receptor, arguing that he "could not imagine natural receptors existing primarily for molecules of a drug that is foreign to the body". This hypothesis gained experimental support when Akil *et al.* [22] found that electrical-stimulation-induced analgesia was partially blocked by the opioid antagonist naloxone thus implying release of an endogenous agonist. Strong support for the hypothesis of an "endogenous opioid ligand" was obtained independently by several groups, who demonstrated the existence of opioid receptors in vertebrates [9-11]. These reports triggered off an intensive search for the endogenous opioid ligands.

The first report of such endogenous opioid activity came simultaneously from two laboratories. Hughes [23] reported the extraction of a material with opioid-like activity from pig brain. He observed inhibition of the electrically induced contractions of the mouse vas deferens by the tissue extract. The opioid-like nature of the active component of the extract was indicated by reversal or blocking of its effect by the opioid antagonist, naloxone. At the same time, Terenius [24] reported an opioid-like

material in aqueous extracts of rat brain. The endogenous material was not only found in the brains of pigs, rats, rabbits and guinea-pigs [25] but also in the cerebrospinal fluid of humans [26].

The endogenous material extracted from pig brain by Hughes [23] was the first one to be identified. Hughes *et al.* [25] showed that it consisted of two penta-peptides, which they called methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu).

Within the same year, Goldstein and co-workers reported the presence of two opioid peptides in pituitary, both inhibited the guinea-pig myenteric plexus-longitudinal muscle preparation in a naloxone-reversible manner, but with evident differences in their pharmacologic behavior. One of these extracted from fresh bovine pituitary was β-endorphin [27], and a second pituitary opioid peptide was the 17-residue dynorphin, now called dynorphin A [28].

All presently known endogenous opioid peptides, that have widespread distribution in the central and peripheral nervous system belong to three families, and each family derives from a separate precursor (see [29]).

1. Pro-opiomelanocortin (POMC) represents the common precursor for β-endorphin, ACTH and several other non-opioid peptides.

2. Proenkephalin A is the precursor for [Met^5]-enkephalin, [Leu^5]-enkephalin and various larger peptides.

3. Prodynorphin provides dynorphin A_{1-17}, dynorphin B_{1-13} and α-neoendorphin.

1.2.2 Multiple Opioid Receptor Subtypes

The first evidence for the existence of multiple opioid receptor subtypes was provided by Martin and co-workers in pharmacological experiments performed in chronic spinal dogs [30,31]. They found that different opioids had different profiles of pharmacologic activity. Moreover, several of the analogues were unable to substitute for each other in the prevention of withdrawal symptoms in dogs chronically treated with one of the other opioids. Based on these findings, Martin distinguished the existence of three types of opioid receptors. He named them for the prototype drugs used in the study: μ for morphine; K for ketocyclazocine and σ for SKF10,047 (N-allylnormetazocine).

Subsequently, Lord *et al.* [32] demonstrated evidence for the existence of different populations of opioid receptor subtypes in mouse vas deferens (MVD) and guinea-pig ileum (GPI) preparations. They found that in isolated GPI, opiate alkaloids were significantly more effective than enkephalins, whereas, in the MVD, it was the enkephalins that exhibited the greater potency. Moreover, the inhibition

of the electrically-stimulated contraction of the MVD by enkephalins was significantly less sensitive to naloxone, an opioid antagonist [33]. These studies suggested that different classes of receptors predominated in these two *in vitro* bioassay systems. The major receptor present in the GPI was called μ, while the receptor in the MVD was called δ.

Further testing of a variety of opioid ligands revealed a correlation between the potency for the displacement of [^{3}H]leucine-enkephalin binding in brain homogenates and the potency in the MVD bioassay, also there was a correlation between potency in the displacement of bound [^{3}H]naloxone from brain homogenates and the inhibition of GPI contractions. The benzomorphan opioids, such as ketocyclazocine and ethylketocyclazocine, were found to be consistently less potent in the MVD than in the GPI. Based on these findings, Lord *et al.* [33] postulated that enkephalins interact with at least two distinct populations of opioid receptor: μ-receptors in GPI, which are antagonized by low levels of naloxone; and δ-receptors in MVD, which require higher concentrations of naloxone for antagonism. Their work also corroborated the existence in the GPI of receptors mediating the effects of the benzomorphan opioids and requiring higher concentrations of naloxone for antagonism, features of the K-receptor subtype postulated by Martin [30]. Similar pharmacological evidence for distinct high-affinity binding sites for morphine and enkephalins was found in brain by Chang and Cuatrecasas [34], who also noted that

many neuroblastoma cell lines expressed only opioid receptors with high-affinity for enkephalins.

Wuster *et al.* [35] proposed yet another opioid receptor, the ϵ-receptor, to explain the high potency of β-endorphin in the rat vas deferens preparation. This preparation responded in an unchanged manner to low concentration of β-endorphin after it had been made highly tolerant to selective μ-, δ-, and K-agonists. The rat vas deferens contains mainly these ϵ-receptor subtypes.

Differences in the regional distribution of these multiple opioid receptor subtypes have also been reported (see [36]). One such study in guinea-pig brain was an autoradiographic survey of brain regions by Goodman and Snyder [37]. By using [^{3}H]ethylketozocine or [^{3}H]bremazocine in the presence of high levels of morphine and enkephalin to block μ- and δ- sites, respectively, it was possible to visualize the K-receptor sites selectively.

To reconcile the large amount of clinical and experimental data, many more subtypes of opioid receptors have been postulated. In fact, the total number of putative opioid receptor subtype numbers nearly a dozen, and even subclasses of each receptor type have already been reported (see [38]). Although very interesting and suggestive, the experimental evidence upon which these receptor types are based is complicated and subject to many difficulties in interpretation. A prominent source

of difficulty is the lack of specific agonists or antagonists for each of these proposed opioid receptor subtypes.

By contrast to this seeming cascade of opioid receptor subtypes, there are some reports suggesting that there may be only one opioid receptor type or at least fewer opioid receptor subtypes than suggested in the literature [39-41]. Zhang and Pasternak [40] used naloxazone, a long-lasting irreversible opioid antagonist, and showed that the analgesic actions of morphine, D-Ala^2-Met^5-enkephalinamide, and β-endorphin were abolished in animals treated with this antagonist. When naloxazone was administered *in vivo* to rats and mice, it selectively inhibited for over 24 h the high-affinity binding of [^{3}H]morphine, [^{3}H]dihydromorphine, [^{3}H]naloxone, [^{3}H]D-Ala^2-D-leu^5-enkephalinamide and [^{3}H]ethylketocyclazocine. By contrast, the low-affinity sites of these ligands were not affected by this drug treatment. Their results, therefore, suggest that all the ligands examined bind with highest affinity to the same population of opioid sites which may be called a receptor site because it is directly related to the analgesic effect of the different agonists; μ, K and δ. Therefore, they concluded that "opiates" and "opioid peptides" have a common analgesic mechanism mediated through a single high-affinity binding site.

There are also some studies indicating the possibility of allosteric interactions between receptor subtypes [42,43], and of multiple modes of ligand interaction with an individual receptor [44,45]. For example, Lee and Smith [45] proposed the

existence of a single universal β-endorphin receptor. Their concept of the "multiple site β-endorphin receptor" stemmed from studies suggesting that β-endorphin interacted with both μ- and δ- receptor sites. β-endorphin is also active in all *in vitro* preparations, including the rat vas deferens where most opioid alkaloids and the natural enkephalin peptides have little or no activity. The β-endorphin receptor was visualized to consist of two sites, one binding with δ-ligands and other with μ-ligands. This receptor could, under appropriate conditions, be activated by a ligand binding at either one of its sites, e.g. D-Ala^2-D-leu^5-enkephalin at δ-site or morphine at μ-site to cause analgesia in the brain, or by β-endorphin, interacting with both sites (δ and μ) simultaneously [45]. Thus, this model predicts interaction between μ- and δ-sites under appropriate conditions as they both form a part of the same macromolecular complex. This model, however, did not deal with the existence of other possible opioid binding sites (*viz.* K, ε, etc.). A similar two-site receptor model has also been proposed by Rothman and Westfall [46] on the basis of binding studies with morphine and enkephalin.

To summarize, there is controversy regarding different types of opioid receptors. Evidence for a multiplicity of opioid receptors has come from many areas, including *in vivo* studies of opioid action, *in vitro* bioassays, binding experiments with selective radioligands, and selective protection and inactivation experiments with alkylating and acetylating agents. However, criteria for differentiating among the subtypes of opioid receptors are unsatisfactory. This is probably because of the lack of sufficiently selective agonists and antagonists.

Recently, more specific synthetic ligands have become available for use (Table 1). These may help resolve the controversy concerning different opioid receptor subtypes. The impression one gets from the literature is that at present there is a growing acceptance of μ-, δ-, and K-receptor subtypes and a non-acceptance or lesser acceptance of the other suggested receptor subtypes. However, the final resolution of the problem of opioid receptor subtypes probably lies in the complete isolation, purification, and structural characterization of all the opioid receptor subtypes.

1.2.3 Opioid Effects on Ion Channels

Opioid effects on ion fluxes have been extensively studied during the last few years, and the use of various electrophysiological techniques have revealed several cellular mechanisms by which opioids depress neuronal activity and neurotransmitter release.

Recently, it has been suggested that μ-, δ- and K- receptors are differentially coupled to ion channels in neuronal membranes: μ- and δ-receptors to potassium channels and K-receptors to calcium channels. Thus, activation of μ- or δ-receptor types causes an increase in a potassium conductance, while activation of K-type causes a reduction in a voltage-dependent calcium conductance [48]. In both cases the net result is a reduction in the rate of neuronal discharge, and a reduction in the amount of transmitter released by nerve cells that happen to bear these receptors.

TABLE 1
Opioid Receptor Subtypes

Receptor Subtype	Selective Agonists	Selective Antagonists
μ	Morphiceptin Tyr-D-Ala-Gly-NMe-Phe-Gly-ol (DAMGO)	Naloxonazine ß-funaltrexamine** Naloxone*
δ	D-Pen^2-D-Pen^5 enkephalin (DPDPE)	ICI-154,129 Naltrindole Naloxone
K	U-50,488H Dynorphin$_{1\text{-}17}$	Nor-binaltorphimine Naloxone
ϵ	β-endorphin	Naloxone
σ^+	Dextromethorphan(?) Haloperidol(?) N-allylnormetazocine (SKF 10,047)	

Note: * Naloxone is a non-selective opioid receptor antagonist.
Naloxone order of potency: $\mu > \epsilon > \delta >$ K.
** Irreversible antagonist. + Sigma receptors may not be exclusively opioid receptors.
Above matter was modified from ref. [47].

Opioid ligands selective for μ-receptors hyperpolarise myenteric [49] and locus coeruleus [50] neurones by increasing the potassium conductance, whereas in dorsal root ganglion (DRG) cells [51], they reduce the duration of Ca^{++}-dependent APs. Opioid ligands selective for δ-receptors hyperpolarised coecal submucosal neurones [52] by increasing potassium conductance and they reduced the duration of Ca^{++}-dependent APs of mouse DRG neurones [53]. By contrast, K-receptor agonists decreased voltage-dependent calcium conductance directly in DRG cells [54,55] and myenteric neurones [56], as their effect persisted even after the potassium current had been blocked.

DRG neurones in cell culture have been shown to contain the three sub-types of opioid receptors *i.e.*, μ, δ and K [55]. In these cells the reduction in the calcium-component of the AP produced by μ- and δ-receptor agonists could be abolished by blocking the potassium current using intracellular caesium or extracellular tetraethylammonium. This shows that the reduction in calcium entry is secondary to an increase in potassium conductance. Also K-receptor agonists depressed the calcium-component of AP of these neurones; however, this effect persisted even after blocking the potassium current, indicating that K-receptor agonists have a direct effect on calcium conductance. Recently, Gross and Macdonald [57], using a voltage-clamp technique, showed that dynorphin A, which is selective for K-receptors, specifically inhibited the N-type calcium channel in mouse DRG neurones in cell culture.

By contrast to mouse DRG cells, in chick DRG cells, Mudge *et al.* [58] found that enkephalins, which are selective for δ-receptors, decreased the calcium-dependent AP. This effect persisted even in the presence of extracellular barium, suggesting a direct inhibition of voltage-dependent calcium conductance by enkephalins.

Recently, a dual modulation of the calcium-component of the AP in mouse DRG neurones by opioid drugs has been reported by Shen & Crain [59], who showed that μ-, δ- and K-receptor agonists in high concentrations (0.1-10 μM) shorten the AP duration, in agreement with previous reports [51,55]. However, interestingly, these agonists in low concentrations [1-10 nM], prolonged the AP duration. These workers went on to show that the prolongation of the AP duration caused by δ- and μ-receptor agonists was due to a decrease in the potassium conductance, whereas that of K-receptor agonists was due to an increase in the voltage-dependent calcium conductance. Thus, these receptors show opposite effects at low and high concentrations. Thus, excitatory and inhibitory responses by opioids may be expressed in the same DRG neurones [59].

The action of opioids on DRG cells is further complicated by the reports of Zieglgansberger and co-workers [60,61]. By contrast to cultured DRG cells, no response to opioids could be detected in mature rat DRG cells. So an alternative hypothesis on the mechanism of action of opioids in the spinal cord was put forward

by Zieglgansberger and co-workers [60,61]. In studies using intracellular recordings from cat dorsal horn neurones *in vivo*, these workers could detect no effect of opioids on the resting membrane potential or APs of these cells. However, the opioids did depress the depolarization and fall in input resistance produced by glutamate. Since glutamate increases the membrane permeability to sodium ions, these workers concluded that opioids block the sodium conductance normally increased by glutamate.

To complicate the picture further, studies from neuroblastoma-glioma hybrid cells (N x G cells) produced different results. In these cell lines, enkephalins have been shown to cause a direct reduction of calcium current [62-65]. Thus, Tsunoo *et al.* [62] found that leucine- and methionine-enkephalins selectively blocked the long-lasting component of the calcium current without affecting the transient calcium current. Also Hescheler and co-workers [63] showed that in N x G cells, opioid receptors were coupled to voltage-dependent calcium channels via G_O protein (a guanine nucleotide regulatory protein having as yet an unknown function). Activation of the opioid receptors in these cells leads to a reduction in calcium current. Recently, δ- as well as μ-receptor agonists have been shown to cause an inhibition of calcium current in human neuroblastoma cells [64].

By contrast to the above studies, Lorentz *et al.* [66] found that in N x G cell lines, morphine activated the calcium current via μ-receptors. The reason for this

discrepancy is unclear; however, it is possible that different culturing conditions may result in different responses to the opioid drugs.

Most studies of the actions and effects of opioids on excitability concentrate on neurones and synapses in the central nervous system, peripheral ganglia and plexuses. However, other studies have shown that opioids, in relatively high concentrations, depress the sodium APs in isolated nerves and skeletal muscles, suggesting the presence of stereospecific opioid receptors on peripheral excitable cells [67-70]. Frank [68,71], first reported the existence of stereospecific opioid receptors in frog skeletal muscle-sartorius muscle. He showed that meperidine, an opioid agonist, blocked APs in this muscle by two mechanisms: (a) nonspecific local anesthetic-like effect; and (b) specific inhibition of the sodium conductance via an opioid drug receptor associated with the muscle fibre membrane. Only the latter effect was blocked by naloxone. Frank proposed that there were opioid receptors on the intracellular surface of the membrane associated with the sodium channels and that opioid activation of these receptors interfered with the opening of the channels normally produced by membrane depolarization. A later study [72], established the stereoisomeric requirement of the opioid-induced late-occurring depression of gNa in frog skeletal muscle fibres.

Hunter and Frank [69] demonstrated the presence of opioid receptors in another peripheral excitable membrane, frog sciatic nerve axon. Analogous to the

earlier observations in frog skeletal muscles [68, 71], it was noted that in the sciatic nerve preparation, meperidine depresses AP production by two distinct mechanisms, a non-specific local anesthetic-like effect and an opioid receptor-mediated effect.

Recently, Frank and Sudha [73] have reported the effects of enkephalins on the excitability of the sciatic and vagus nerves. In these preparations, enkephalins produced naloxone-reversible depression of AP. However, enkephalins were effective only when applied intracellularly, suggesting the presence of opioid receptors on the inner surface of the cell membranes of peripheral vertebrate nerve axons.

1.2.4 **Opioid Receptors and Signal Transduction**

The binding of opioids to their 'receptors' has been extensively studied. However, the molecular events that take place subsequent to the activation of opioid receptors by an agonist have not yet been clearly elucidated.

Collier and Roy [74], first demonstrated the role of cAMP in opioid actions. They showed that morphine and other opioids inhibited prostaglandin E-stimulated cAMP formation in rat brain homogenates. This inhibition was seen at concentrations of opioids comparable to those required for analgesia and it was antagonized by naloxone.

Soon after the above original observation, opioid inhibition of adenylate cyclase and consequently cAMP formation, by both μ- and δ-receptors was amply demonstrated in mammalian brain [75, 76] and the neuroblastoma x glioma hybrid cell line [77, 78]. Further, it has been shown that opioid receptors are uncoupled from adenylate cyclase inhibition by treatment of neuroblastoma x glioma hybrid cells with pertussis toxin, which uncouples receptor-mediated inhibition of adenylate cyclase [79].

Although, G_i (guanine nucleotide binding protein: G-protein) mediated inhibition of adenylate cyclase remains the best characterized biochemical effect of opioid-receptor activation, electrophysiological studies suggest that other mechanisms also account for opioid inhibition in many neuronal systems. Hescheler and co-workers [63] have shown that in a neuroblastoma x glioma hybrid cell line, opioid receptors are coupled to voltage-dependent calcium channels via G_o protein and activation of the receptors leads to a reduction in calcium current [63].

Cyclic 3, 5-guanosine monophosphate (cGMP) levels in different regions of the brain too have been shown to be affected by opioids in a stereospecific and naloxone-reversible manner. In slices of rat striatum, leu-enkephalin, met-enkephalin, morphine and levorphanol, but not dextrorphan, enhanced the accumulation of cGMP with a concomitant decrease in cAMP levels [80].

Aghajanian and coworkers [81] have suggested that the μ-receptors in locus coeruleus neurones are coupled to adenylate cyclase via G_i protein. Activation of these receptors would thus be expected to cause a reduction in cAMP. Membrane permeable analogues of cAMP were able to reverse the opioid-induced hyperpolarization caused by an increase in potassium conductance [82]. Interestingly, North *et al.* [83,84] were unable to detect any effect of forskolin, a direct activator of adenylate cyclase, on the opioid response of locus coeruleus neurones and also of guinea-pig submucous plexus. But intracellular application of GTP[S] (a nonhydrolysable analog of GTP-Guanosine 5-(γ-thio)-triphosphate) produced an agonist-dependent uncoupling of the inhibitory response, causing a prolonged hyperpolarization after the agonist washout. Further, opioid inhibition of locus coeruleus neurones is not seen after pretreatment with pertussis toxin [81]. Taken together, these results suggest that a G protein indeed mediates the opioid activation of K^+ channels in these preparations, but through a pathway independent of cAMP-dependent protein kinase. Opioid inhibition in guinea-pig myenteric neurones has been previously shown to be insensitive to elevation in cAMP levels [85].

Thus, at present, biochemical and electrophysiological studies do not correlate well regarding the involvement of specific G proteins to opioid-receptor activation. However, it is interesting to note that most of the responses to opioid receptor activation, so far studied, show involvement of a pertussis toxin-sensitive G protein.

1.3 CALCIUM-OPIOID ANTAGONISM

It has long been known that calcium counteracts the analgesic effect of morphine [86-88]. For example, Hano *et al.* [87] reported that intracisternal administration of calcium antagonized opioid-induced analgesia. Furthermore, EDTA (ethylenediaminetetraacetic acid) inhibited the antagonistic effect of calcium on the analgesic response to opioids [87]. In fact, EDTA alone produced a weak analgesic effect, which was diminished by calcium [87]. The ability of calcium to antagonize opioid effects was enhanced by the ionophores A23187 or X537A [88,89], which are known to increase the permeability of membranes to calcium. Molecules able to chelate Ca^{++} [e.g. EDTA] or to antagonize calcium fluxes (e.g. lanthanum, D-600, verapamil) potentiate the analgesic action of morphine [88], opioid peptides [90], and of several benzomorphans classified as K-receptor agonists [91].

In particular, studies of the interactions of opioids with calcium transport mechanisms in biological membranes have revealed a calcium-antagonistic effect of opioids. Several workers have shown that opioids inhibit depolarization-induced $^{45}Ca^{++}$ synaptosomal accumulation in a naloxone-reversible, dose-dependent manner [92-94]. Since K^+- stimulated Ca^{++} uptake by synaptosomes primarily represents voltage-dependent Ca^{++} influx, Kamikubo *et al.* [94] have suggested that morphine inhibits depolarization-dependent Ca^{++} uptake into synaptosomes by inhibiting the voltage-dependent Ca^{++} influx.

An interaction between opioids and the brain calcium content seems to play a basic role not only in the acute effects of opioids but also in the chronic effects of opioids. Physical dependence on morphine has been shown to be associated with a large increase in the content of Ca^{++} in the brain of mice and rats. This increased content of Ca^{++} could be antagonized by naloxone and during naloxone precipitated withdrawal syndrome, these elevated Ca^{++} levels quickly returned towards normal values [95]. Molecules that modify Ca^{++} fluxes, such as lanthanum, when injected intracerebroventricularly reduced most of the signs of morphine abstinence syndrome [96].

It has been shown that the number of [^{3}H]nitrendipine binding sites, and therefore, probably of voltage-operated calcium channels, are significantly increased in the brain of morphine-dependent mice [97]. Furthermore, the calcium channel blockers verapamil and nimodipine have been shown to suppress the morphine withdrawal syndromes in rats [98]. Several workers have reported the effects of calcium channel blockers and stimulants on the morphine abstinence syndrome [98-100], suggesting a role for calcium channels in opioid withdrawal syndrome.

There are a number of reports suggesting that Ca^{++} is involved in the actions of opioids other than those mediating analgesia. Thus, calcium has been shown to antagonize the morphine-induced depression of the electrically stimulated ileum [101-103]. Calcium has also been shown to antagonize the depression of neuromuscular

transmission produced by morphine in the mouse vas deferens [104]. Using intracellular recording techniques, Rohani and Frank [105] showed that increasing the intracellular Ca^{++} concentration in the frog sartorius muscle antagonizes the depressant effect on excitability produced by opioids acting on an intracellularly oriented opioid receptor.

Recently, several electrophysiological studies have shown that opioids block voltage-dependent calcium channels in several preparations (this part has already been discussed in detail in section 1.2.3), e.g., spinal dorsal root ganglion cells [57, 58] and neuroblastoma x glioma hybrid cells [62-65]. Thus, activation of δ-receptors by enkephalins has been shown to reduce calcium conductance in neuroblastoma x glioma hybrid cells [62] and activation of δ- as well as μ-receptors has been shown to cause inhibition of calcium currents in human neuroblastoma cells [64].

Thus, several lines of evidence indicates that Ca^{++} is involved in the acute and chronic actions of opioids. Calcium also seems to play an important role in the opioid dependence and abstinence syndrome.

1.4 CALCIUM CHANNELS

The important role of calcium in the regulation of many cellular functions is now widely recognized. Calcium controls an array of biological processes, including cell growth and muscle contraction, as well as neurotransmitter and hormone release (see [106]). The mechanisms involved in these functions are as yet incompletely understood. A diverse array of Ca^{++} transporting systems function to maintain the steep concentration gradient between extracellular Ca^{++}, known to be in the millimolar range, and intracellular Ca^{++}, which can vary between 0.1-10 μM, depending on the state of the cell. Specific and distinct pathways exist by which Ca^{++} enters and exits cells (see [107]).

The major entry pathway for Ca^{++} in many cell types is via plasma membrane calcium channels. The opening of these calcium channels enables Ca^{++} ions to move passively across the membrane down their electrochemical gradient into the cell. Ca^{++} channels are important for supplying Ca^{++} to many types of cells, particularly to excitatory cells such as muscle and nerve, in which they are postulated to play a role in excitation-contraction coupling and neurotransmitter release, respectively (see [106]).

Different types of Ca^{++} channels are known to exist and are characterized by fundamental differences in the mechanism governing their opening and closing.

Some Ca^{++} channels are voltage-dependent and open in response to an appropriate membrane depolarization. Within this category, there are subclasses of Ca^{++} channels that differ in their voltage sensitivities, kinetic properties, and pharmacological sensitivities (see [108]). These voltage-dependent calcium channels are discussed in detail in the following section.

The second category of Ca^{++} channels includes channels that are operated through receptor-dependent mechanisms. These channels are often referred to as receptor-operated Ca^{++} channels, and are opened in response to activation of an associated receptor (see [108,109]). Little is known about the functional characteristics of receptor-operated Ca^{++} channels or their link to membrane receptors. Examples include channels in smooth muscle that are opened by activation of ATP receptors [110] and parathyroid hormone activated Ca^{++} channels in osteosarcoma cells [111]. Although, the receptor-operated Ca^{++} channels have received much less scrutiny than have voltage-operated Ca^{++} channels, they are currently the subject of extensive investigation.

Other types of Ca^{++} channels include leak channels and stretch-sensitive channels (see [112]). The Ca^{++} influx through leak channels is a consequence of the large electro-chemical gradient tending to drive Ca^{++} into the cell [113]. This Ca^{++} influx mechanism has been termed as "channel" for reasons of convenience, but it is unlikely to occur via a gated channel of classical structure.

The mechanical effects on vascular smooth muscle are believed to promote Ca^{++} influx through stretch-sensitive channels that probably differs from Ca^{++} influx through voltage- or receptor-operated channels [112]. However, little is known about the functional characteristics of these stretch-sensitive calcium channels.

1.4.1 Voltage-Dependent Calcium Channels

Voltage-dependent Ca^{++} channels open in response to an appropriate membrane depolarization. Three different types of voltage-dependent Ca^{++} channels have been defined by electrophysiological and pharmacological techniques (see [108,114]).

1. L-type, high-voltage activated or slow Ca^{++} channel, which conducts a long-lasting current of large conductance.
2. T-type, low-voltage activated or fast Ca^{++} channel, which is activated at low voltages and is characterized by transient currents with small conductance.
3. N-type, which is neither T- nor L-type, but is activated at relatively high voltages and conducts a relatively transient current of intermediate size. N-channels have been described only in neurons [115].

Different properties of T-, L- and N-type Ca^{++} channels are summarized in Table 2. One or more types of voltage-dependent Ca^{++} channels may exist in a particular cell type. For example, in sensory neurons of dorsal root ganglia, all the three channel types co-exist [117]. In skeletal muscle, both L- and T- type, but not N-type, channels have been observed [118].

Compared with the fast Na^{+} channels, the voltage-dependent slow Ca^{++} channels are kinetically much slower. These slow Ca^{++} channels operate over a less-negative (more depolarized) voltage range; *i.e.*, their threshold potential and the inactivation voltage range are higher (less negative). A "three state" model, similar to the Hodgkin-Huxley model for the fast Na^{+} channel [127], has been proposed for voltage-dependent slow Ca^{++} channel. The three states are: resting, active and inactive. Depolarization to the threshold potential activates the slow Ca^{++} channel from resting (nonconducting) state to the active (conducting) state. With continued depolarization, the channel passes into the inactive (nonconducting) state. The recovery process upon repolarization returns the channel from the inactive state back to the resting state (see [128]).

1.4.2 Calcium Channel Antagonists and Agonists

Insights into the properties and functions of Ca^{++} channels were mainly obtained by the discovery of agents that have selective effects on Ca^{++} channels. Not only are

TABLE 2

Properties of L-, N- and T-types of voltage-dependent calcium channels

Ca^{++} channel type → Properties	L (slow)	N (neuronal)	T (transient)
Activation range	Positive to -30 mV	Positive to -30 mV	Positive to -70 mV
Channel conductance	Large	Moderate	Small
Dihydropyridine sensitivity	Yes	No	No
Sensitivity to Ca^{++}-agonists (eg. Bay K 8644)	Yes	No	No
Sensitivity to isoprenaline	Yes	No	No
Sensitivity to ω-conotoxin	Yes (neuron) No (muscle)	Yes	No

NOTE: Above matter was modified from ref. (116).

some of these agents clinically useful but also they have been, and continue to be, useful in understanding biochemical and electrophysiological properties of Ca^{++} channels.

Drugs that selectively or specifically inhibit Ca^{++} channels are collectively referred to as "Ca^{++} channel antagonists" or "Ca^{++} channel blockers", and those capable of activating Ca^{++} channels, such as Bay K8644 and CGP 28392 are referred to as "Ca^{++} channel agonists" or "Ca^{++} channel activators" [119]. These drugs have been the subjects of extensive reviews [119-121].

Ca^{++} channel antagonists consist of at least three distinct classes of drugs: 1,4-dihydropyridines, phenylalkylamines and benzothiazipines, represented by the prototypes-nifedipine, verapamil, and diltiazem, respectively. These drugs act by binding with high-affinity to distinct but allosterically interacting receptor sites. Most of the drugs developed as Ca^{++} channel antagonists appear to have selective effects on the voltage-dependent slow Ca^{++} channels [119].

Ca^{++} channel antagonists show signifcant tissue selectivity both pharmacologically and therapeutically (see [119]). The Ca^{++} channel antagonists exhibit different binding affinities, depending on the membrane potential and the frequency of channel opening (or "use"). For example, Ca^{++} antagonists of dihydropyridine series have been shown to act with higher affinity on the depolarized

membrane preparation [119, 126]. Thus, in skeletal muscle the $K_{0.5}$ for (+)PN 200-110, a 1,4-dihydropyridine type antagonist, to inhibit Ca^{++} channels in depolarized cells was 0.15 nM, whereas the $K_{0.5}$ for (+)PN 200-110 to inhibit the same channels in polarized cells was significantly higher, 13 nM [122]. These findings are generally interpreted to indicate that dihydropyridines bind better to the inactivated form of the channel than to the resting form (see [119]).

Use-dependent blockade, in which the inhibitory activity of a drug increases with increasing frequency of stimulation, reflects a preferential drug interaction with, or access to, an activated- or inactivated-channel state. This occurrence has been described for verapamil, diltiazem and their analogs [123-125]. These use-dependent actions are also influenced by the level of the membrane potential between stimuli-hyperpolarization and depolarization, reducing and increasing, respectively, the extent of use-dependent blockade (see [119]).

Some dihydropyridines (Bay K 8644 and CGP 28392) have been shown to increase rather than decrease Ca^{++}-influx through voltage-sensitive slow Ca^{++} channels. These drugs bind to the same dihydropyridine receptor that binds to Ca^{++} antagonists like nifedipine. Dihydropyridine-type Ca^{++} channel activators also show significant voltage-dependent interactions with Ca^{++} channels (see [119]).

1.4.3 Regulation of Calcium Channels

Ca^{++} channels in many cells are subject to regulation by neurotransmitters by at least two different types of processes. The first to be recognized, involves regulation of the channels by second messenger mediated phosphorylation/dephosphorylation dependent events. The second, involves regulation of channel activity by GTP-binding proteins (G proteins) (see [108]).

The first Ca^{++} channel recognized to be regulated by neurotransmitters was the L-type Ca^{++} channel present in heart. Norepinephrine acting through beta-adrenergic receptors, increases the slow-inward current, while acetylcholine via muscarinic receptors, reverses the effect of norepinephrine and decreases slow-inward current [129,130]. These effects of norepinephrine and acetylcholine are believed to involve a cAMP-dependent regulation of the L-type Ca^{++} channels. Any agent that elevates cAMP levels in cardiac cells, including beta-adrenergic receptor agonists, modulates the activity of the cardiac L-type Ca^{++} channels by increasing their probability of opening (see [108]).

Additional evidence for the regulatory role of cAMP has been demonstrated by the use of GTP analogue, GPP(NH)P, which directly and irreversibly activates adenylate cyclase, induced Ca^{++}-dependent slow APs in chick heart cells [131]. Iontophoric application of cAMP into dog purkinje fibers and guinea-pig ventricular

muscle cells induces Ca^{++}-dependent slow APs in the injected cells [132]. cAMP has been shown to enhance directly slow calcium current in isolated single cardiac cells [133]. Taken together, these results support the hypothesis that cardiac L channels are regulated by the cAMP-dependent phosphorylation of the channel itself or a regulatory protien. Although good evidence exists to suggest that muscarinic effects are due to decreased cAMP [134], other regulatory processes may be involved since acetycholine is believed to regulate other second messenger systems in cardiac muscle [135]. L-type Ca^{++} channels in skeletal muscle [136], are also modulated positively by processes that appear to involve cAMP-dependent phosphorylation. cAMP has also been reported to have stimulatory effects on T-type Ca^{++} channels in frog skeletal muscle [136].

Protein kinase C has also been shown to be involved in the regulation of Ca^{++} channels (see [108]). Thus, activators of protein kinase C, such as tumor promoting phorbol esters, have been shown to inhibit L- and N-type Ca^{++} channels in embryonic chick and mouse DRG neurons, respectively [137,57].

Direct regulation of Ca^{++} channels by GTP-binding proteins (G-protein) has also been identified in several tissues (see [138]). Thus, inhibition of voltage-dependent Ca^{++} channels by receptors coupled to G-proteins has been demonstrated with norepinephrine, $GABA_B$ and opiate receptors in chick DRG neurones [139], rat DRG neurones [140] and NG 108-15 neuroblastoma x glioma hybrid cells [63], respectively. In all the above examples cited, the G-protein involved was pertussis toxin-sensitive.

Participation of the α-subunit of G_O protein in the opiate receptor-mediated regulation of Ca^{++} channels in neuroblastoma cells was shown by infusing purified G_O or its α-subunit into the cells [63]. Recently, the cardiac L-type channel has been shown to be directly activated by G_S (the G protein that stimulates adenylate cyclase) [141].

Thus, regulation of Ca^{++} channels by various mechanisms seems to be far more complicated than was indicated in the original studies of regulation of cardiac Ca^{++} channels by norepinephrine.

1.4.4 Molecular Structure of the Dihydropyridine-Sensitive Calcium Channel

Skeletal muscle t-tubules have the greatest density of dihydropyridine binding sites [142]. Accordingly, investigators have concentrated on purifying these receptors on the premise that it is the L-type Ca^{++} channel and is related to Ca^{++} channels in other tissues (see [143]).

Purified preparations of skeletal muscle have been incorporated into artificial membranes, and electrophysiologic and pharmacologic studies have confirmed the presence of active Ca^{++} channels [144-146]. The L-type Ca^{++} channel is thought to be a multisubunit complex comprosed of α_1-, α_2-, β-,γ- and δ-subunits (Fig.2). There is no uniform agreement on the exact subunit composition or the existence of δ-subunit, which is linked by disulfide bond(s) to the α_2-subunit. The α_1-subunit and the β-subunit

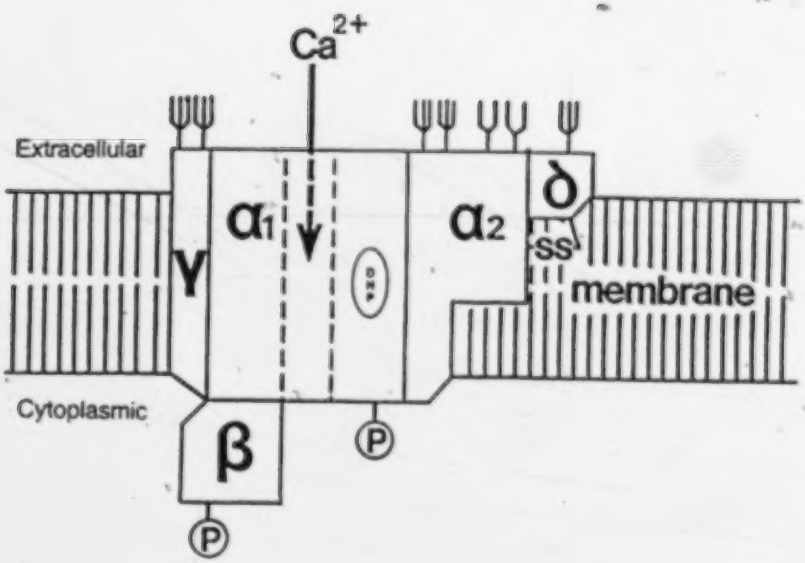

FIG. 2 Proposed model for calcium channel structure. Sites of cAMP-dependent phosphorylation (P), glycosylation, and interaction with the membrane are illustrated. The α_1 subunit, which contains the calcium antagonist binding sites, cAMP-dependent phosphorylation sites, and the largest hydrophobic domains, is proposed to be the central ion channel-forming component of the complex.
(Note: Above figure was modified from ref. [147].)

are phosphorylated by protein kinases. Using photoaffinity probes, the α_1-subunit was identified as the receptor for the nifedipine- and verapamil-type drugs (see [143]).

The amino acid sequence of dihydropyridine receptor (α_1- subunit of the L-type voltage-dependent Ca^{++} channel) has recently been deduced (1873 amino acids) [148]. Just as for the Na^{+} channel, there are four motifs, each containing six membrane spanning regions, S1 to S6. The minimum subunit assembly necessary for voltage-dependent Ca^{++} channels has not been yet determined.

1.4.5 Skeletal Muscle Calcium Channels

Like other excitable cells, skeletal muscle fibers have voltage-dependent Ca^{++} channels. These channels are localized in the t-tubule system of the cell membrane [118,149,150].

The voltage-activated Ca^{++} currents are mediated by two separate Ca^{++} channel subtypes. One subtype mediates transient- (T-) Ca^{++} current that is, rapidly activated at membrane potentials more positive than -65 mV and is not sensitive to organic Ca^{++} channel blockers. The second type mediates a long-lasting Ca^{++} current that is, activated at membrane potentials more positive than -30 mV and is blocked by organic Ca^{++} channel blockers [118]. The above two subtypes of Ca^{++} channels are also observed in mammalian skeletal muscle cells (myoballs) in culture [151]. The basis for

the decline of inward Ca^{++} current is somewhat controversial, and has been attributed either to a Ca^{++} depletion from the t-tubule lumen [149] or to a voltage-dependent gating process [152].

1.4.6 Role of Calcium Channels in Excitation-Contraction Coupling

Contraction of a skeletal muscle fiber is initiated after an AP depolarizes the membrane of the transverse-tubules (t-tubules), a system of infolded sarcolemmal membrane, reaching into the interior of the fiber. Somehow, depolarization of the t-tubular membrane results in release of Ca^{++} from the internal stores of the sarcoplasmic reticulum, a system of specialized internal membranes that can sequester large concentrations of Ca^{++} ions. The released Ca^{++} activates the contractile machinery of the fiber. The link between t-tubule depolarization and release of internal Ca^{++} resulting in mechanical activation has been termed "Excitation-Contraction Coupling" [153].

Ca^{++} entry through voltage-dependent Ca^{++} channels plays an essential role in excitation-contraction (EC) coupling in cardiac and smooth muscle cells [154,155]. Conversely, contractile force of skeletal muscle is often considered to be independent of Ca^{++} influx. The Ca^{++} involved in skeletal muscle contraction is believed to come mainly, if not entirely, from stores in sarcoplasmic reticulum [156].

The mechanism of skeletal muscle EC coupling is the current focus of intense research efforts. Several hypotheses have been proposed to explain EC coupling in skeletal muscle, but the one that has withstood the test of time is the "trigger Ca^{++}" hypothesis [159-162]. According to this hypothesis, there are Ca^{++} ions bound to the intracellular surface of the t-tubular membrane, which are referred to as "trigger Ca^{++}" ions. When the t-tubular membrane is depolarized these "trigger Ca^{++} " ions are released and diffuse across the triadic junctional space to the terminal cisternae of the sarcoplasmic reticulum, where they cause the release of a large quantity of Ca^{++}, that is, Ca^{++}-induced Ca^{++}-release. The Ca^{++} ions released from the sarcoplasmic reticulum interact with the contractile proteins to initiate the mechanical response or twitch. However, tonic responses produced by prolonged depolarizations, such as high-potassium contractures, require the entrance of extracellular Ca^{++} ions for EC coupling. Thus, in studies using isolated skeletal muscle fibers, it was shown that in a Ca^{++}-free solution, high-potassium contractures were eliminated in few seconds but it took several minutes to block twitches [163]. Voltage-dependent Ca^{++} channels are mainly concentrated in the t-tubules and most of them do not have enough time to open during a twitch but would open during longer depolarizations [157,158]. This led to the idea that the Ca^{++} ion flux through these channels occurs during long-depolarizations such as potassium contractures but not during twitches [164]. Twitches mainly utilize "trigger Ca^{++} ". Using various Ca^{++} channel blockers, it was further shown that these drugs could block high-potassium contractures specifically, without blocking the twitch [164-166].

Recently, inositol 1,4,5-triphosphate (IP_3) produced by the hydrolysis of membrane bound phosphatidylinositol 4,5-diphosphate has been proposed as a chemical transmitter in EC coupling in skeletal muscle [167,168]. The ability of IP_3 to release Ca^{++} ions from sarcoplasmic reticulum preparations isolated from skeletal muscle is a disputed point, with some workers finding this effect [169] and others not [170]. Studies concentrated on the ability of applied IP_3 to produce mechanical responses in skinned muscle fibers are controversial. Some reported a mechanical response produced by IP_3 (169, 171), while others did not observe such a response (170, 172). However, even when the response was obtained, it was much slower and always high concentrations were needed to produce this response.

The "trigger Ca^{++}" and "IP_3" mechanisms for EC coupling, discussed above, are essentially chemical transmission type of mechanisms. More elaborate mechanisms have been proposed for EC coupling in skeletal muscle [173]. These involve a mechanical connection between the t-tubular membrane and the terminal cisternae of the sarcoplasmic reticulum. The ryanodine receptor, that is, Ca^{++}-release channel of the sarcoplasmic reticulum, has been proposed to form "bridge" or "feet" that anatomically connects t-tubules and sarcoplasmic reticulum. The mechanical link is suppose to involve voltage-dependent charge movements as proposed some years ago by Schneider and Chandler [174].

It has been reported that there are far more dihydropyridine binding sites than the functional Ca^{++} channels in skeletal muscle [175] concentrated in the t-tubules. These dihydropyridine receptors are suggested to be "voltage sensors" responsible for generating intramembrane charge movements in the t-tubules upon depolarization [176]. These charge movements are blocked by Ca^{++} antagonists of dihydropyridine-type [177]. Thus, dihydropyridine receptor, that is, voltage sensor, has been suggested to be associated functionally with the "feet" processes, which cross the triadic junction and have been identified as ryanodine receptors [178]. Together, these two receptors are thought to couple the electrical activity in the t-tubules to the release of Ca^{++} ions from the terminal cisternae of sarcoplasmic reticulum [173]. However, this theory ignores considerable evidence supporting roles for extracellular and t-tubular bound Ca^{++} ions in EC coupling in skeletal muscle [163-166]. Another problem with this theory is the observation that the Ca^{++} channel blockers, which greatly depress the charge movements, do not inhibit EC coupling during a twitch [164,165,179].

2. STATEMENT OF THE PROBLEM

During a prior study of the mechanism for opioid-induced twitch potentiation in our laboratory, it was observed that methadone, an opioid agonist, at a concentration which potentiated the twitch, also reduced the size of K^+ contractures [180]. As these contractures are produced by calcium ions that enter the muscle fibers via the voltage-dependent slow calcium channels, it was suggested that methadone was blocking these channels. In view of this finding, it was of interest to see if opioids, in general, produce a block of voltage-dependent slow calcium channels in the frog skeletal muscles, and if so, to investigate the possible involvement of a stereospecific opioid receptor in this response. K^+ contractures and Ca^{++}-dependent slow APs were the two responses used to study the effects of opioids on voltage-dependent slow calcium channels in frog skeletal muscle.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Solutions

The compositions of the experimental solutions used for specific experiments were as foliows:

- Choline-Ringer's solution (mM): choline chloride, 111.8; KCl, 2.47; $CaCl_2$, 1.08; $NaHC0_3$, 2.38; NaH_2P0_4, 0.087; and glucose, 11.1.
- Sodium-Ringer's solution (mM): NaCl, 111.8; KCl, 2.47; $CaCl_2$, 1.08; $NaHC0_3$, 2.38; NaH_2PO_4, 0.087; glucose, 11.1; and d-tubocurarine, 0.1 mg/mL. Tubocurarine was employed to eliminate possible neuromuscular effect. It has been shown to block specifically the transmitter action of acetylcholine at the neuromuscular junction [209].
- Isotonic KCl (High-K^+) solution: 123 mM KCl and 1.17 mM $CaCl_2$.
- K^+-glucuronic acid (High-K^+) solution: 123 mM of D-glucuronic acid - K^+ salt and 1.17 mM $CaCl_2$. This solution had a $[K^+]$ x $[Cl^-]$ product of 288, which was the same as the choline-Ringer's solution. The potassium salt of the D-glucuronic acid was made with KOH and D-glucuronic acid (Sigma Chemical Company, St. Louis, MO).
- Cl^--free, Na^+-free and high-K^+ solution (mM): glucose, 11.1; sucrose, 200; K^+-acetate, 20; and Ca^{++}-acetate, 3.

Cl^--free, high-K^+-sodium Ringer's solution (mM): glucose, 11.1; K^+-acetate, 10.0; Ca^{++}-acetate, 1.08; Na^+-acetate, 111.8; and d-tubocurarine, 0.1 mg/mL.

Cl^--free, high-K^+ solution (mM): K^+-acetate, 123; Ca^{++}-acetate, 1.08.

In experiments involving the use of elevated Ca^{++} levels (5 mM), the required amount of Ca^{++} was added to the appropriate experimental solution without reducing other ions.

In experiments involving the use of 10 mM K^+ solution, the required amount of KCl was added to the choline- or sodium-Ringer's solution without reducing other ions. Experiments involving the use of 25 and 40 mM K^+ solution, the required amount of KCl was added by isotonic replacement of NaCl.

All solutions were prepared fresh for each experiment, and the pH was adjusted at room temperature to be between 7.2 and 7.4.

3.1.2 **Drugs**

Drugs used in this study were:

(a) Morphine hydrochloride - May and Baker.

(b) Methadone hydrochloride - May and Baker.

(c) Fentanyl citrate - McNeil Labs.

(d) Etorphine hydrochloride - Reckitt and Colman.

(e) Met-enkephalin (acetate salt) - Sigma Chemical Company.

(f) Leu-enkephalin (acetate salt) - Sigma Chemical Company.

(g) $Dynorphin_{1-13}$ (acetate salt) - Sigma Chemical Company.

(h) Morphiceptin hydrochloride - Sigma Chemical Company.

(i) D-Ala-Leucine-Enkephalinamide (acetate salt) - Sigma Chemical Company

(j) Des-Tyr-D-Ala-Leucine-Enkephalinamide (acetate salt) - Sigma Chemical Company

(k) Naloxone hydrochloride - Endo Labs Inc.

(l) Levorphanol tartrate - Hoffmann - La Roche.

(m) Dextrorphan hydrochloride - Hoffmann - La Roche.

(n) Meperidine hydrochloride - Winthrop Labs Inc.

(o) Caffeine - Nutritional Biochemicals Corporation.

(p) Nifedipine - Sigma Chemical Company.

(q) Diltiazem hydrochloride - Sigma Chemical Company.

(r) U-50,488H - The Upjohn Company.

(s) Nor-binaltorphimine - Research Biochemicals Inc.

(t) Haloperidol - McNeil Labs.

Drug solutions were prepared routinely by dissolving the required amount of the substance directly into the appropriate experimental solution. Exceptions to the general procedure were as follows:

Caffeine solution (2.36 mM) was prepared by dissolving the requisite amount of the base in concentrated hydrochloric acid and then making up to the desired volume with choline-Ringer's solution. The resulting caffeine solution was buffered to pH 7.2 by the addition of sodium bicarbonate.

Nifedipine, nor-binaltorphimine and haloperidol were first dissolved in few mL of ethanol, and then they were diluted to the desired volume with the appropriate experimental solution. In control experiments, ethanol concentrations equivalent to those in the 10 μM drug solutions were found to have no effect on K^+ contractures, twitches and Ca^{++}-dependent slow APs.

3.2 METHODS

3.2.1 Tension Studies

3.2.1.1 Muscle Preparation

The extensor longus digiti IV (toe) muscles of the frog *Rana pipiens* were used in all experiments involving tension studies. Experiments were routinely carried out during all the seasons of the year. Each frog was decapitated and pithed and the muscle was removed. After removal, the muscle with each of its tendons tied to a silk thread was placed in a glass petri dish containing choline- or sodium-Ringer's

solution. The silk threads were then wrapped around two plastic pegs which were stuck to the inner surface of the dish close to its circumference and at diametrically opposite ends. This helped in positioning the muscle in the petri dish and facilitated further dissection. With the aid of a dissecting microscope (Wild-Heerbrugg, Switzerland) the muscle was freed from connective tissue and fascial membranes. All muscles were allowed to equilibrate for a period of at least 30 min following dissection in the physiologic solution before an experiment was commenced.

3.2.1.2 **Muscle Chamber**

The muscle chambers used for tension recordings were constructed from glass barrels of syringes of 3 or 5 mL capacities. The toe muscle was mounted in the chamber between a glass hook and a strain gauge. The proximal end of the muscle was secured to the glass hook near the bottom of the bath and the distal end was attached to the arm of the strain gauge positioned above the bath, by means of the silk threads. The position of the strain gauge was adjusted using a micromanipulator. The solution in the chamber was changed by draining at the bottom and by introducing fresh solution at the top. The resting tension of the muscle was adjusted so that muscle came back to a vertical position without assistance when the bath solution was put back into a previously drained bath. To remove the high-K^+ solution, the bath was flushed twice about 1 min apart with Ringer's solution.

3.2.1.3 **Tension Recording**

Isometric tensions were recorded by means of pixie transducers (Endevco Model 8121A) in a wheatstone bridge configuration. The output was recorded either with a pressurized ink spray recorder (Gould Brush 2200) or a digital stripchart recorder (Dianachart). All experiments were carried out at room temperature ($20^{o}C$). Muscles were allowed to equilibrate for a period of at least 30 min after dissection before an experiment was started.

3.2.1.4 **General Experimental Protocols**

The toe muscles were excised and mounted as described above. For studying opioid effects on K^+ contractures, two or more control responses of the muscle to a high-K^+ solution (123 mM) were initially obtained. Each of the exposure periods to the above solution was kept short, *viz.* 15-30 s, so as to minimize muscle swelling by any electrolytic imbalance. After each test with elevated K^+, the muscle was kept in a choline-Ringer's solution containing 2.47 mM K^+ usually for 15 min before another test was performed. After obtaining the 'control' responses with high-K^+ solution, the responses of the muscle to elevated K^+ were afterwards recorded in the presence of the opioid, and they were compared with the 'control' responses obtained earlier.

In experiments involving twitch recording, the sodium-Ringer's solution bathing the muscle was continuously bubbled with a gas mixture of 99.5% 0_2 and 0.5% $C0_2$. By employing this particular gas mixture, the pH of the sodium-Ringer's solution remained at approximately 7.2. Supramaximal rectangular pulses 0.5 ms in duration were applied once every 30 s to the muscles by means of two ring electrodes of platinum, situated at the top and bottom of the solution in the bath. The tension generated was recorded with a pressurized ink spray recorder (Gould Brush 2200). If the height of the control twitches remained steady for 30 min, the tissue was considered suitable for an experiment. After obtaining the 'control' twitches, the responses of the muscle were recorded in the presence of the drug and they were compared with the 'control' responses obtained earlier.

3.2.1.5 **Analysis**

Measurements were taken from the recordings made on chart paper. Results were routinely expressed as a percentage of the 'control' (100%) response. Since it was only possible to obtain one measurement of particular drug per tissue, the effects at various drug concentrations were normalized and averaged from different experiments to construct the dose-response curves. Where necessary, the significance of the difference between two means was assessed with Student's *t* test; a level of significance of $P < 0.05$ was used.

3.2.2 Electrophysiological Studies

3.2.2.1 Muscle Preparation

The sartorius muscles of the frog *Rana pipiens* were used in all experiments involving electrophysiological studies. Experiments were carried out during all the seasons of the year. The sartorius muscle along with a portion of the pelvic girdle containing the proximal attachment of the muscle was carefully dissected and removed from a decapitated and pithed frog. The muscle was then placed in a dissecting dish and, with the aid of a dissection microscope, was freed from connective tissue and fascial membranes. The whole muscle was mounted horizontally in a Sylgard bath containing Ringer's solution in such a way that the deep surface of the muscle lay uppermost. This was important, as the superficial surface is covered with a layer of connective tissue which hinders the insertion of microelectrodes. For final mounting, the muscle was stretched approximately 20% of slack length over two glass posts and then firmly pinned down in the bath. The stretch prevented damage to the microelectrode tips upon movements of the muscle and also minimized movement artifacts. The bath was illuminated from below and viewed from above with a binocular dissecting microscope (Wild-Keerbrugg, Switzerland).

3.2.2.2 Stimulation and Recording System

A small proportion of the muscle fibres were stimulated by an extracellular, bipolar, platinum-filled pore electrode (tip diameter = 0.4 mm) placed on the surface of the muscle 5-20 mm from the recording electrode. For recording normal sodium action potentials, the muscle fibres were stimulated by 0.5 ms single pulses delivered through a stimulus isolation unit (WP Instruments, model 305B). While for recording Ca^{++}-dependent slow action potentials, fibres were stimulated by pulses of 60-100 ms duration. Occasionally a 200 ms duration pulse was used but in most instances a 70 ms pulse was used.

Resting membrane and action potentials of the frog sartorius muscle fibres were recorded intracellularly using conventional glass capillary microelectrodes (10-40 M ohm resistance) filled with 3 M KCl. A broad-band electrometer (WP Instruments, Model M-4) was used to measure membrane potentials. The output of the electrometer was connected to an oscilloscope (Nicolet 3091 Digital Oscilloscope). The reference electrode consisted of a chlorided silver wire, formed into a spiral, and placed in the solution bathing the muscle.

The records from the digital oscilloscope were stored on floppy disks and later analysed and pictures of action potentials drawn using a Compaq Deskpro 286 computer and a Roland DGPR-1212A printer. All programs for transmitting, storing and analysing data were written in Turbo Pascal by Dr. G.B. Frank.

3.2.2.3 Recording Electrodes

Recording electrodes were made from open-ended glass capillaries (Fisher Scientific Co.) having 1.5-2 mm outside diameter. The microelectrodes were drawn by a glass microelectrode puller (PN-3 Narishige Scientific Instrument Lab. Ltd., Japan). These microelectrodes were then mounted on microscope slides with a rubber band wrapped around them and placed in a cylindrical dish with their tips downward. The dish was then filled with methanol and placed in a vacuum dessicator. The methanol was allowed to boil for about 5 min by reducing the pressure in the dessicator. With this procedure, almost all the electrodes were completely filled with methanol. The slides with the electrodes were then transferred successively into two cylindrical dishes each containing distilled water and they were left in each dish for 4-5 h Finally, the electrodes were placed in 3 M KCl solution and left there overnight before use. Only microelectrodes having resistances of about 10-40 M ohm were used. A solid state volt-ohm-microamp meter (Danameter, Dana Lab Inc., Irvine, California) was used to measure the resistance of the microelectrodes.

3.2.2.4 General Experimental Protocols

Electrical events were recorded from (usually) 3-5 surface fibers over short time periods (about 5 min overall). After equilibration period of 30 min, a few

sodium action potentials were recorded. Then sodium-Ringer's solution was replaced by Cl^--free, Na^+-free, high-K^+ solution to record Ca^{++}-dependent slow APs. After a 30 min equilibration period, 'control' responses (Ca^{++}-dependent slow APs), usually 6 to 8 were obtained. Then the muscle was exposed to the drug dissolved in Cl^--free, Na^+-free, high-K^+ solution and further electrical events were obtained usually after every 15 min.

Opioid effects on high-K^+ induced membrane depolarization were studied using sucrose gap apparatus similar to one used by Frank and Sudha [75]. Frog toe muscle was pulled through the holes in the four rubber membranes of a five-chambered sucrose gap apparatus. The width of each chamber was 3 mm. The drugs and high-K^+ solutions were added to the end chamber and the change in membrane potential was recorded across the sucrose gap.

3.2.2.5 **Analysis**

The effect of any given concentration of an opioid on action potential was evaluated by comparing the area under the action potential (Ca^{++}-dependent slow APs) with the mean area of the control response (expressed as % of predrug control area). The exact area under the action potential, eliminating the stimulus artifact, was analyzed using Turbo Pascal Program. Where necessary, the significance of the difference between two means was assessed with Student's *t* test; a level of significance of $P < 0.05$ was used.

4. RESULTS

4.1 K^+ CONTRACTURES

4.1.1 Introduction

It is well established that K^+ contractures in skeletal muscle require extracellular Ca^{++} ions and are abolished after exposure to a Ca^{++}-free solution [181-183]. These K^+ contractures are produced by the influx of extracellular Ca^{++} through voltage-dependent slow calcium channels and they can be blocked by various organic calcium channel blockers [164-166, 184,185]. The existence of a voltage-dependent inward Ca^{++} current located in the transverse tubules further supports the role for external Ca^{++} ions in the development of K^+ contractures [186, 187]. Recently, Cota and Stefani [118] found that two inward Ca^{++} currents could be detected in twitch muscle fibres of the frog: a fast- and a slow-inward current. It was suggested that the fast Ca^{++} current is active during a single twitch, while the slow Ca^{++} current might be activated during K^+ contractures [118,188]. All of the above evidence strongly supports the concept that the mobilization of Ca^{++} from extracellular sources through voltage-dependent slow calcium channels occurs during K^+-depolarization induced excitation-contraction coupling in skeletal muscle [159, 162, 164].

4.1.2 Effect of non-peptide opioids on K^+ contractures

It was earlier reported from our laboratory that methadone, in a concentration that had little or no effect on the resting membrane potential, inhibited K^+ contractures in frog skeletal muscle. As these contractures are produced by the influx of extracellular Ca^{++} ions through voltage-dependent slow calcium channels located in the t-tubules, it was concluded that methadone blocked these channels [180]. The experiments described in this section were carried out to extend this study further by investigating the effects of a variety of opioids on the high-K^+ contracture response of the frog toe muscle.

Typical depolarization contractures obtained with the high-K^+ (123 mM) solution and the inhibition of these contractures produced by the opioid agonist methadone (10^{-5} M) are shown in Fig. 3. In a similar manner, contractures evoked by the high-K^+ solution were inhibited by several other non-peptide opioid agonists and the antagonist naloxone in a concentration-dependent manner (Table 3).

It took 2-4 h to develop equilibrium effects with the sub-blocking concentrations of the opioids. With higher concentrations, the contractures were blocked in less than 2 h and recovery to 40-80% of control was observed in 1 h after the drugs were removed from the bath solution. With lower concentrations of the drugs, the recovery was more rapid.

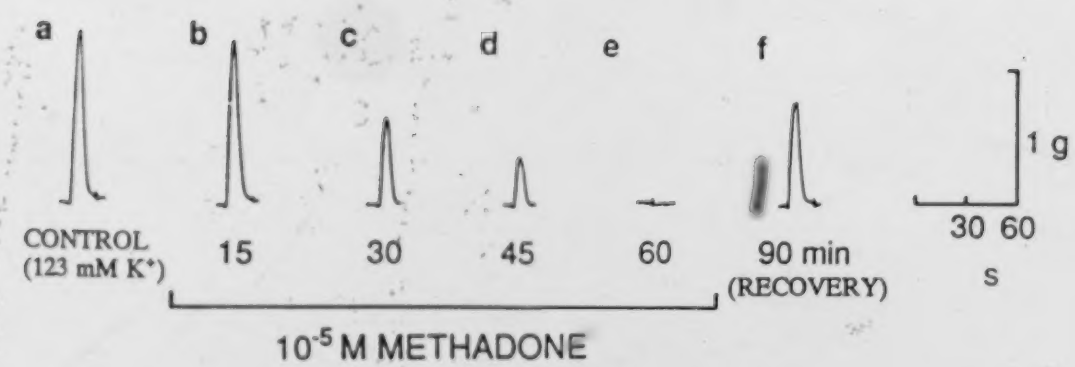

FIG. 3 Effect of methadone on 123 mM K^+-induced contractures of a frog toe muscle. Tests were made every 15 min. Horizontal line under the records indicates exposure to methadone (10^{-5} M) in choline-Ringer's solution. Muscle was exposed to methadone (10^{-5} M) till complete block of K^+ contracture was obtained (i.e., for 60 min). Exposure to the high-K^+ solution was kept for 15-30 s. Times below records indicate the time after the start of exposure to methadone. (a) Control response; (b-e) tests during exposure to methadone. The muscle was placed back in a drug-free solution immediately following test shown in (e). (f) Recovery test.

TABLE 3

Depression of K^+ contractures by various non-peptide opioids in the isolated toe muscle of frog

Drug	Maximum Inhibition (%)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-10} M
Morphine	-	97.4 ± 2.6 (4)	-	71.8 ± 9.7 (4)	64.7 ± 6.9 (9)	27.8 ± 1.7 (4)
Methadone	-	98.9 ± 1.1 (5)	-	91.6 ± 6.5 (5)	80.2 ± 7.4 (6)	36.5 ± 1.8 (5)
Meperidine	-	94.5 ± 4.3 (4)	-	73.4 ± 5.4 (5)	58.3 ± 8.1 (9)	29.4 ± 3.0 (7)
Levorphanol	-	92.7 ± 7.4 (3)	63.6 ± 4.5 (4)	-	22.5 ± 2.7 (3)	-
Dextrorphan	-	96.7 ± 3.3 (3)	64.9 ± 2.9 (5)	-	28.4 ± 5.1 (3)	-
Naloxone	91.2 ± 8.6 (3)	70.4 ± 8.1 (5)	32.3 ± 9.2 (4)	-	8.8 ± 3.0 (6)	3.2 ± 1.8 (4)
U-50,488H	-	97.2 ± 2.8 (5)	81.2 ± 2.8 (13)	50.0 ± 5.2 (6)	-	-
Fentanyl	-	90.3 ± 4.9 (4)	-	-	-	-
Etorphine*	-	100.0 (1)	-	77.2 (1)	-	-

NOTE: Means ± S.E.M. ---, not tested. Number in bracket = number of experiments

* Due to limited quantity of the compound only two experiments were carried out.

These results were used to construct the log concentration-effect curves for methadone, morphine, meperidine and naloxone shown in Fig. 4. As it was only possible to obtain one measurement per tissue, the effects of various drug concentrations were averaged to construct the curves. Curves constructed in this manner greatly reduce the estimated slope of the log concentration-% effect curve below the "true" slope for these curves [189], thereby leading to a reduced estimate of the effect of the higher drug concentrations. Thus, at 10^{-8} M, a complete block (*i.e.*, a 100% effect) was produced in one of nine tests with morphine, in two of six tests with methadone and in two of nine tests with meperidine, while the mean inhibition effects were 64.7, 80.2 and 58.3%, respectively. The opioid effects on the K^+ contracture were found to be quite variable with respect to the degree of inhibition obtained at a given concentration of the drug using muscles from different batches of frogs, but no seasonal effect was noticed.

4.1.3 **Effect of opioid peptides on K^+ contractures**

Interestingly, while the non-peptide opioids mentioned above were effective in reducing K^+ contractures in very low concentrations, various opioid peptides produced little or no effect on these contractures (Table 4). Thus, dynorphin in a concentration as high as 10^{-5} M, showed almost no inhibition of K^+ contractures, when tested for 3 h (Fig. 5). Morphiceptin in same concentration (10^{-5} M) could produce only 20.7% inhibition of high-K^+ contractures (Table 4).

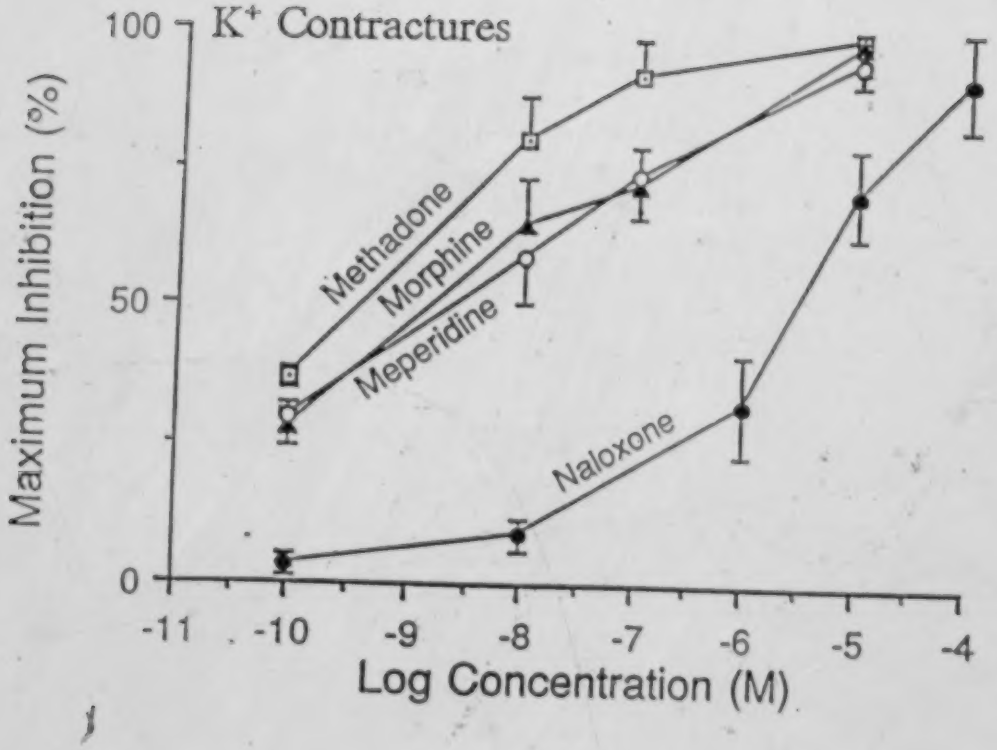

FIG. 4 Inhibition of K^+ contractures produced by methadone (⊡), morphine (▲), meperidine (o), and naloxone (●). The points are the means with one (±) standard error. When a standard error is not shown it was less than the size of the symbol. In these experiments, only one value was obtained per experiment.

TABLE 4

Effect of various opioid peptides on the K^+ contractures of frog toe muscle

Drug	Maximum Inhibition (%)		
	10^{-4} M	10^{-5} M	10^{-6} M
Morphiceptin	38.0 ± 5.4 (3)	20.7 ± 1.4 (3)	12.0 ± 3.4 (3)
Leu-enkephalin	35.4 ± 5.0 (3)	30.2 ± 6.3 (5)	22.3 ± 2.4 (3)
D-Ala^2-leucine enke--phalinamide	---	16.3 ± 1.6 (3)	---
Dynorphin	---	11.9 ± 2.8 (4)	7.5 ± 3.1 (4)

NOTE: Means ± S.E.M.
---, not tested
Number in bracket = number of experiments

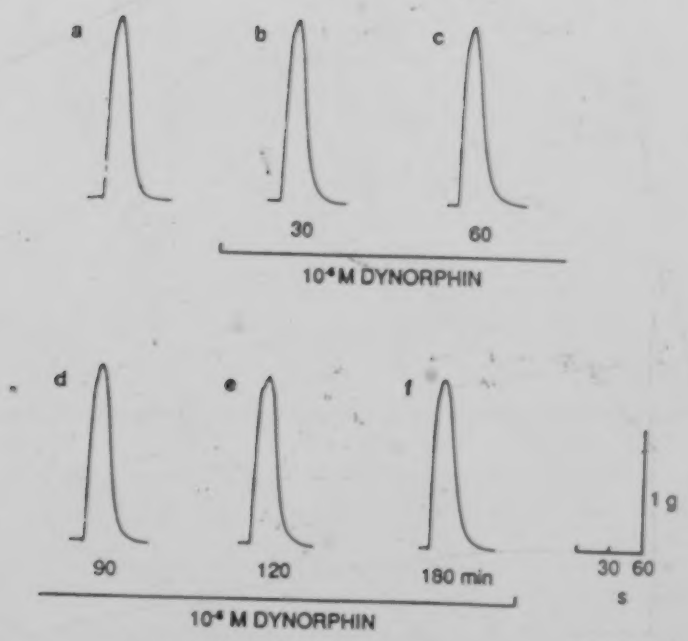

FIG. 5 Lack of effect of dynorphin, an opioid peptide, on 123 mM K^+-induced contractures of a frog toe muscle. Tests were made every 15 min. Horizontal line under the records indicates exposure to dynorphin (10^{-5}M) in choline-Ringer's solution. Times below records indicate the time after the start of exposure to dynorphin. (a) Control response; (b-f) tests during exposure to dynorphin.

4.2 INVESTIGATION OF THE POSSIBLE INVOLVEMENT OF A STEREOSPECIFIC OPIOID RECEPTOR IN THE K^+ CONTRACTURE DEPRESSION PRODUCED BY OPIOIDS

4.2.1 Studies with opioid stereoisomers

Levorphanol, a potent opioid analgesic, and dextrorphan, its analgesically inactive isomer, exhibited no meaningful differences in their ability to produce a depression of K^+ contractures (Fig. 6). Thus, levorphanol and dextrorphan (10^{-6} M, each) produced 63.6% and 64.9% inhibition of K^+ contractures, respectively (Table 3). This result demonstrated that the effective opioids produced a nonstereospecific blockade of K^+ contractures.

4.2.2 Studies with opioid antagonists

To test further the lack of involvement of a stereospecific opioid receptor in the depression of K^+ contractures by the non-peptide opioids, an opioid antagonist naloxone was used. When naloxone was tested alone in concentrations ranging from 10^{-8} M to 10^{-4} M, it produced inhibition of K^+ contractures (Fig. 4 and Table 3). Since a lower concentration of naloxone (10^{-10} M) did not produce any measurable effect on K^+ contractures, 10^{-10} M naloxone was chosen to test for antagonism. In one instance, 10^{-8} M was used. However, no antagonism by naloxone was observed

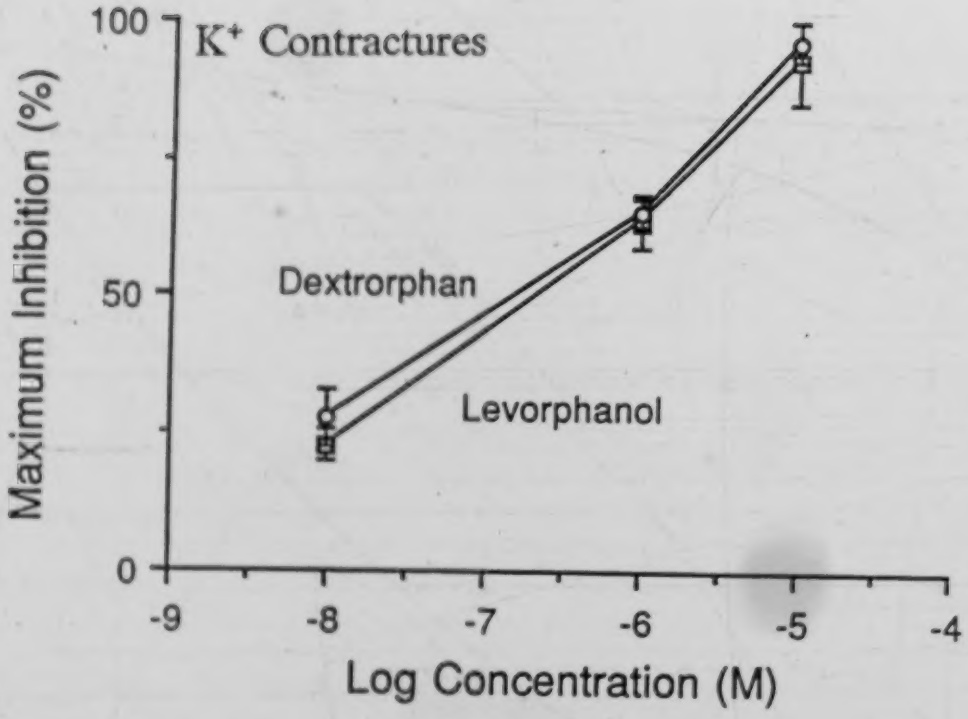

FIG. 6 Inhibition of K^+ contractures (123 mM) produced by different concentrations of stereoisomers; levorphanol (□), a potent opioid analgesic, and dextrorphan (○), an inactive isomer.

(Table 5). The only noticeable effect of naloxone addition was an increase in contracture inhibition.

Naloxone did not antagonize the K^+ contracture depression produced by U-50,488H, an opioid agonist specific for the K-receptor subtype (Table 6). Also norbinaltorphimine, an opioid antagonist specific for the K-receptor subtype did not antagonize the inhibition of the K^+ contractures produced by U-50,488H (Table 6, Fig. 7). Thus, norbinaltorphimine in concentrations ranging from 10^{-10} M to 10^{-6} M did not produce any antagonism of the U-50,488H (10^{-6} M and 10^{-7} M) induced inhibition of K^+ contractures.

TABLE 5

Depression of K^+ contractures produced when low concentrations of naloxone were combined with opioid agonists

Agonist (concen) + Naloxone (concen)	Maximum inhibition(%)	Difference from control values* (%)
Methadone (10^{-8} M) + Naloxone (10^{-8} M)	96.9 ± 1.6 (3)	+ 16.7
Methadone (10^{-8} M) + Naloxone (10^{-10} M)	84.4 ± 7.9 (3)	+ 4.2
Morphine (10^{-8} M) + Naloxone (10^{-10} M)	70.7 ± 10.0 (6)	+ 6.0
Meperidine (10^{-8} M) + Naloxone (10^{-10} M)	67.5 ± 7.7 (6)	+ 9.2
Dextrorphan (10^{-6} M) + Naloxone (10^{-10} M)	67.4 ± 2.4 (3)	+ 2.5
Levorphanol (10^{-6} M) + Naloxone (10^{-10} M)	70.4 ± 5.7 (3)	+ 6.8

NOTE: Means ± S.E.M.
Values in parentheses are the number of experiments.
* See Table 3 for control values (i.e., % inhibition in absence of naloxone).

TABLE 6

Lack of antagonism of naloxone and norbinaltorphimine on the K^+ contracture inhibition produced by U-50,488H in the isolated toe muscle of frog

U-50,488H concentration (M)	Antagonist concentration (M)	Maximum inhibition (%)	Difference from control values* (%)
U-50,488H (10^{-6})	Naloxone (10^{-5})	86.3 ± 3.2 (3)	+ 5.1
U-50,488H (10^{-6})	Naloxone (10^{-6})	81.0 ± 1.5 (3)	- 0.2
U-50,488H (10^{-6})	Naloxone (10^{-9})	80.0 ± 5.4 (3)	- 1.2
U-50,488H (10^{-7})	Naloxone (10^{-7})	53.0 ± 1.2 (3)	+ 3.0
U-50,488H (10^{-6})	Norbinaltorphimine (10^{-6})	86.0 ± 4.9 (3)	+ 4.8
U-50,488H (10^{-6})	Norbinaltorphimine (10^{-8})	88.0 ± 2.1 (3)	+ 6.8
U-50,488H (10^{-6})	Norbinaltorphimine (10^{-5})	90.0 ** (1)	+ 8.8
U-50,488H (10^{-7})	Norbinaltorphimine (10^{-10})	53.7 ± 5.0 (3)	+ 3.7
U-50,488H (10^{-7})	Norbinaltorphimine (10^{-7})	56.8 ± 6.7 (3)	+ 6.8

NOTE: Means ± S.E.M.
Values in parentheses are the number of experiments
* See Table 3 for control values (i.e., % inhibition in absence of antagonist).
**Due to limited quantity of norbinaltorphimine only one experiment was performed using a high concentration (10^{-5} M) of drug.

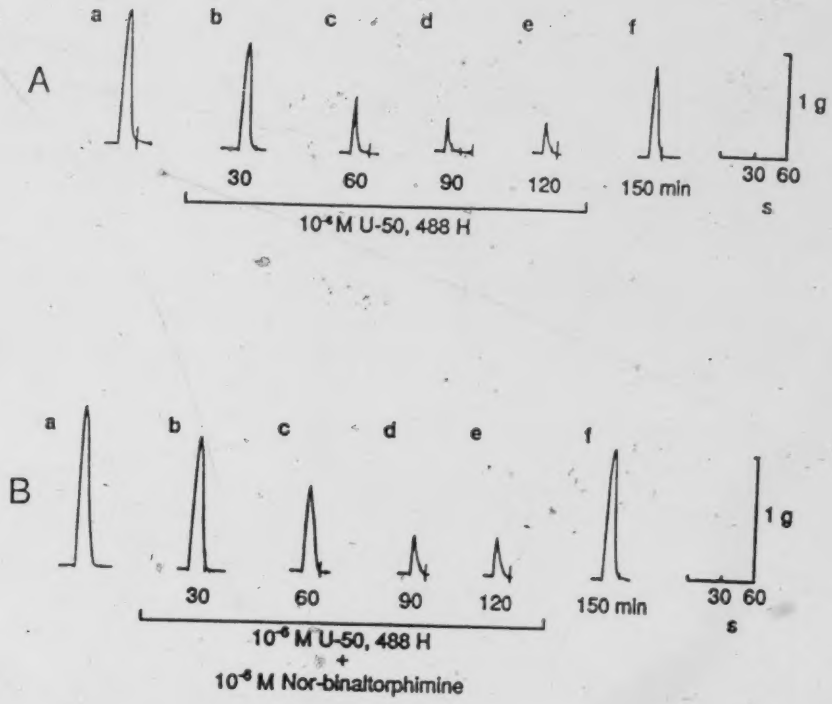

FIG. 7 A: Effect of U-50,488H (10^{-6} M) on the K^+ contractures of frog toe muscle. Tests were made every 15 min. Exposure to drug is shown by the horizontal line below the records. (a) Control response; (b-e) tests during exposure to U-50,488H; (f) recovery response after 30 min into a drug-free solution. B: Effect of U-50,488H (10^{-6} M) on the K^+ contractures of frog toe muscle in the presence of nor-binaltorphimine (10^{-6} M). Tests were made every 15 min. (a) control response; (b-e) tests during exposure to U-50,488H + nor-binaltorphimine; (f) recovery response after 30 min into a drug-free solution.

4.3 ANTAGONISM OF THE OPIOID BLOCK PRODUCED BY RAISING THE EXTRACELLULAR CALCIUM CONCENTRATION

To determine whether Ca^{++} ions could reverse the opioid block of K^+ contractures, experiments were performed in which the effect of an elevated extracellular calcium concentration (5 mM) on the block produced by 10^{-5} M morphine was tested. The results obtained in the experiment illustrated in Fig. 8 were typical of the four experiments performed. Morphine (10^{-5} M) caused a complete block of the K^+ contracture after 1.5 h in a choline-Ringer's solution containing 1.08 mM Ca^{++} and this block was completely reversed when the bathing solution Ca^{++} concentration was raised to 5 mM. The complete block of the K^+ contracture was restored when the bathing solution Ca^{++} concentration was reduced back to 1.08 mM (n=4, Fig. 8).

Dextrorphan (10^{-6} M) and levorphanol (10^{-6} M) caused 64.9% and 63.6% contracture inhibitions, respectively, in choline-Ringer's solution containing 1.08 mM Ca^{++} (Table 3). In a separate experiment with each, when the bathing solution Ca^{++} concentration was 5 mM, these drugs produced only a 20% (dextrorphan) and a 30% (levorphanol) inhibition of the K^+ contracture.

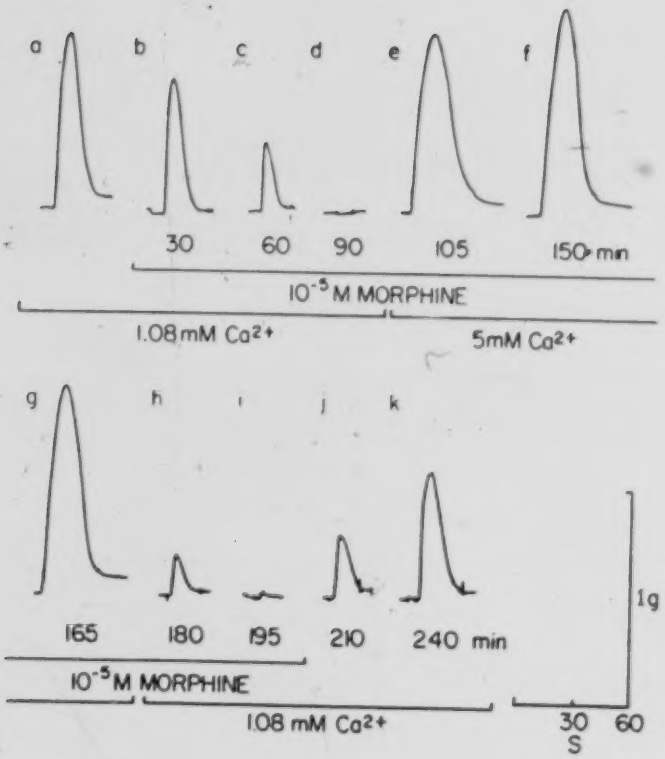

FIG. 8 Reversal of the morphine (10^{-5} M) block of 123 mM K^+-induced contractures in a frog toe muscle produced by raising the extracellular calcium level to 5 mM. Exposure to morphine is shown by the horizontal line below the records. Times listed below the records indicate the time after the start of exposure to morphine. Bathing solution calcium concentration is indicated by a second lower horizontal line. During exposure to morphine tests were made every 15 min. (a) Control response. Immediately after (d) the Ca^{2+} concentration was raised to 5 mM, immediately after (g) it was lowered to 1.08 mM again, and immediately after (i) the morphine was removed from the solution bathing the muscle.

4.4 EFFECT OF OPIOIDS ON CAFFEINE-INDUCED CONTRACTURES

To determine whether non-peptide opioids were depleting the intracellular Ca^{++} stores or inhibiting the release of Ca^{++} from these intracellular stores, experiments were performed in which the effects of the opioids on caffeine-induced (2.36 mM) contractures were studied. Caffeine-induced is thought to produce contractures by sensitizing the sarcoplasmic reticulum to Ca^{++}-induced Ca^{++} release [190, 191]. The results obtained in an experiment with morphine (10^{-7} M) are shown in Fig. 9. In this experiment, morphine (10^{-7} M), which produced a 93% block of the K^+ contracture, did not reduce the maximum tension of the caffeine-induced contracture. Three experiments each were conducted with morphine (10^{-5} M and 10^{-7} M) and essentially similar results were obtained, *i.e.*, a 65-100% inhibition of the K^+ contractures but little (14% inhibition) or no effect on the caffeine-induced contractures (n=3, each). Methadone in a concentration of 10^{-5} M and 10^{-7} M, produced an average 100% (n=3) and 85% (n=3) inhibition of the K^+ contractures, respectively, but showed very little (maximum 18% inhibition) effect on caffeine-induced contractures.

4.5 EFFECT OF OPIOIDS ON THE K^+-INDUCED MEMBRANE DEPOLARIZATION

Using a sucrose gap apparatus, experiments were performed to see the effect of opioids on the K^+-induced membrane depolarization. Methadone (10^{-5} M), which

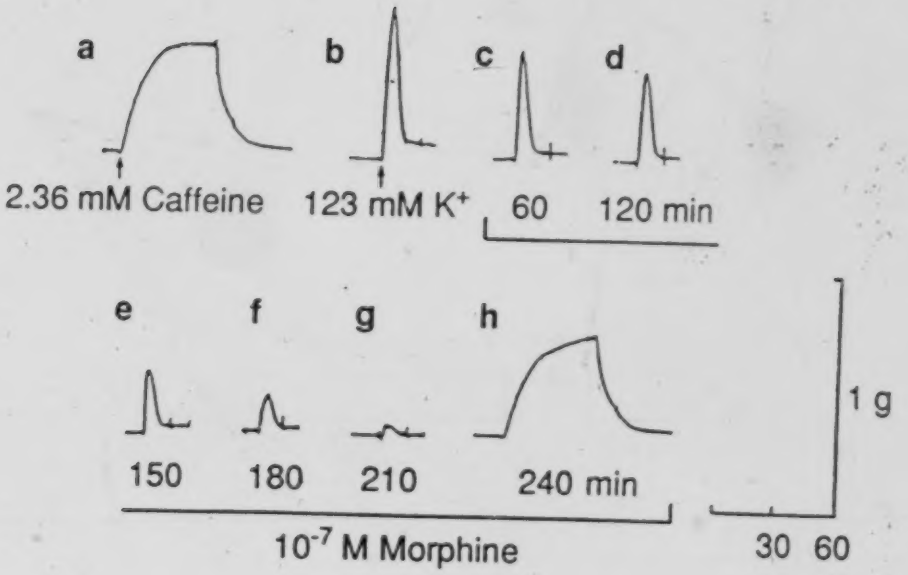

FIG. 9 Effects of morphine on K^+- and caffeine-induced contractures of a frog toe muscle. Tests were made every 15 min. Horizontal line under the records indicates exposure to morphine (10^{-7} M) in choline-Ringer's solution. Times listed below the records indicate the time after the start of exposure to morphine. (a and b) Control responses to caffeine and KCl, respectively; (c-g) K^+ contractures; and (h) a caffeine contracture during the exposure to morphine.

completely blocked the K^+ contractures in 60-90 min (Table 3), did not produce any effect on the K^+-induced membrane depolarization when tested after a 90 min exposure to the drug (n=3, Fig. 10). A longer 3 h exposure to 10^{-8} M methadone, also had no effect on the depolarization produced by the high-K^+ (123 mM) solution. Ninety-min exposures to the opioid agonists, morphine (10^{-5} M, n=3) and levorphanol (10^{-5}M, n=3) also were found to have no effect on the K^+-induced membrane depolarization.

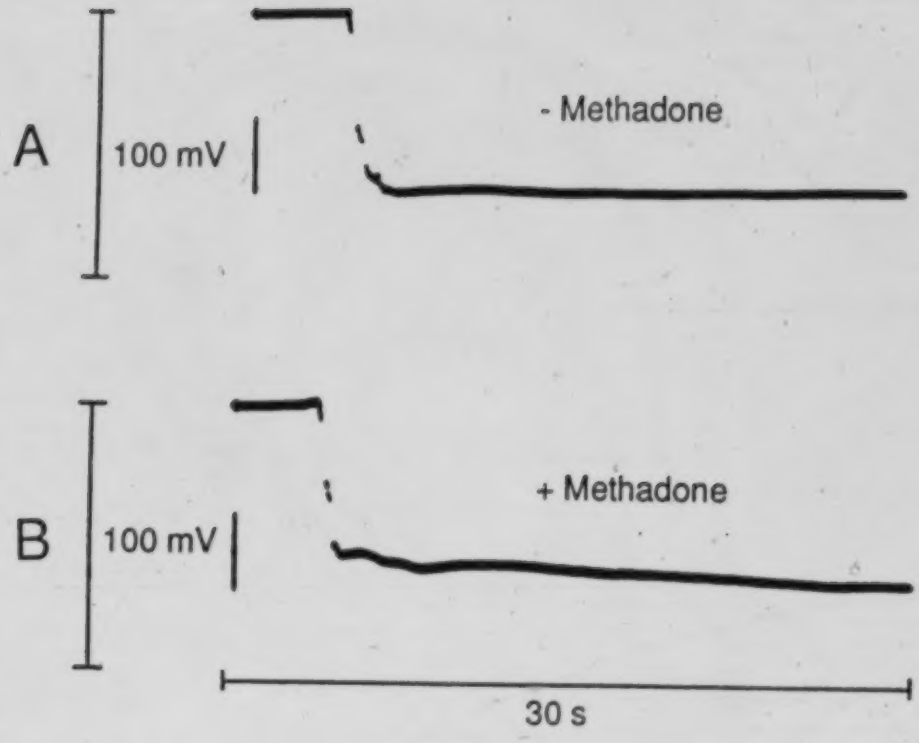

FIG. 10 Lack of effect of methadone (10^{-5} M) on the K^+-induced membrane depolarization. Oscilloscope recordings of membrane potentials obtained using a sucrose gap apparatus. (A) 123 mM K^+ without methadone; (B) 123 mM K^+ after a 90 min exposure of the same frog toe muscle to the methadone. The scale at the left of each record equals 100 mV. Each sweep lasted 30 s. Depolarization is shown as a downward deflection.
(NOTE: These experiments were carried out by Dr. T. Subrahmanyam Sudha)

4.6 Ca^{++}-DEPENDENT SLOW ACTION POTENTIALS

4.6.1 Induction and Ca^{++}-dependency of slow APs

Ca^{++}-dependent slow APs in frog skeletal muscle can be induced by using a Cl^--free (acetate substituted), Na^+-free (sucrose substituted), high-K^+ (20 mM) solution [192]. Fig. 11 shows an example of induction and Ca^{++}-dependency of slow APs in frog skeletal muscle. A normal skeletal muscle fast AP (Na^+-AP) is shown in Fig. 11A. After replacing the normal Ringer's solution, with the Cl^--free, Na^+-free, high-K^+ (20 mM) solution, the membrane depolarized to approximately -40 mV from a usual resting potential of -90 mV in Ringer's solution and normal Na^+-APs could no longer be generated. However, Ca^{++}-dependent slow APs could be produced.

As $[Ca]_o$ was raised from 1.08 mM to 3 and 5 mM (Fig. 11 - B, C and D, respectively), the amplitude and the duration of the slow APs increased. Data of this type, pooled from a number of experiments, are summarized in Table 7. Amplitude (in mV) and duration (in s, measured at 50% amplitude) of the slow APs are plotted as a function of $[Ca]_o$ (log scale) in Fig. 12. The mean amplitude of the slow APs increased in a linear manner from approximately 34 mV in 1.08 mM Ca^{++} to 71 mV in 5 mM Ca^{++} (Fig. 12, filled circles). The average slope of the AP amplitude *vs.* log $[Ca]_o$ line was 47.8 mV/decade, which is higher than the theoretical slope of 29 mV/decade as predicted from the Nernst equation at 21°C for a divalent cation. However, it should be noted that this value (47.8 mV) was obtained using only three

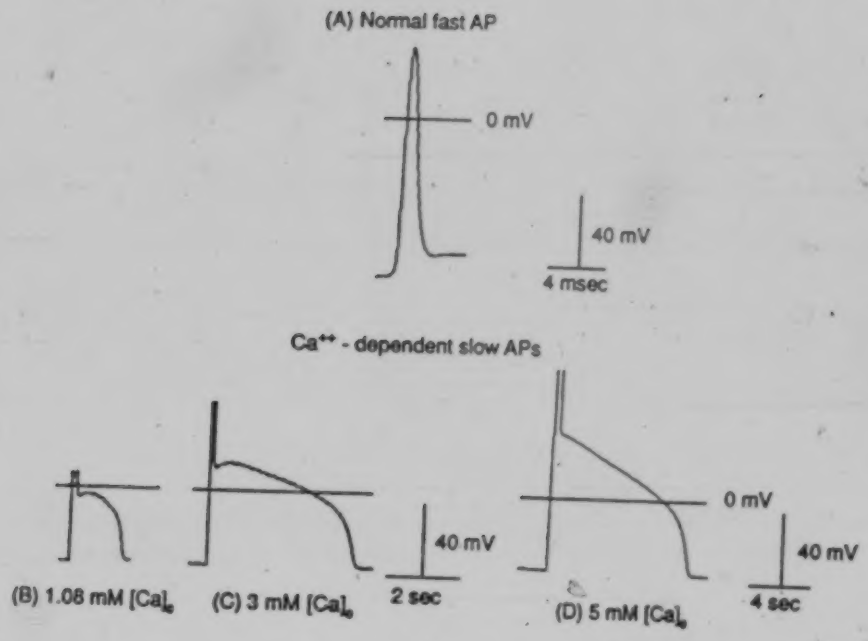

FIG. 11 (A): Normal fast AP obtained using normal Ringer's solution. (B), (C) and (D): Dependency of Ca^{++}-dependent slow APs on $[Ca]_o$. Slow APs were obtained using a Na^+-free (sucrose substituted), Cl^--free (acetate substituted), high-K^+ (20 mM) medium. Increasing $[Ca]_o$ from 1.08 mM (B) to 3 mM (C) and 5 mM (D) produced an increase in amplitude and duration of the slow AP. A prominent stimulus artifact is visible at the start of the slow AP in panels B, C, and D. The upper horizontal lines in each panel give the zero potential level.

TABLE 7

Effect of $[Ca]_o$ on the slow APs in the isolated sartorius muscle of frog

$[Ca]_o$ (mM)	Ca^{++}-dependent slow APs*		
	Duration** (s)	Amplitude (mV)	Area (mV-s)
1.08	0.7 ± 0.1	33.6 ± 2.2	17.2 ± 1.8
3	3.7 ± 0.4	57.8 ± 2.1	158.3 ± 10.1
5	7.6 ± 0.9	70.9 ± 3.0	380.2 ± 24.8

NOTE: Mean ± S.E.M.

* Duration, amplitude and area of the slow APs, eliminating the stimulus artifact, were measured using the turbo pascal program.

** Measured at 50% amplitude.

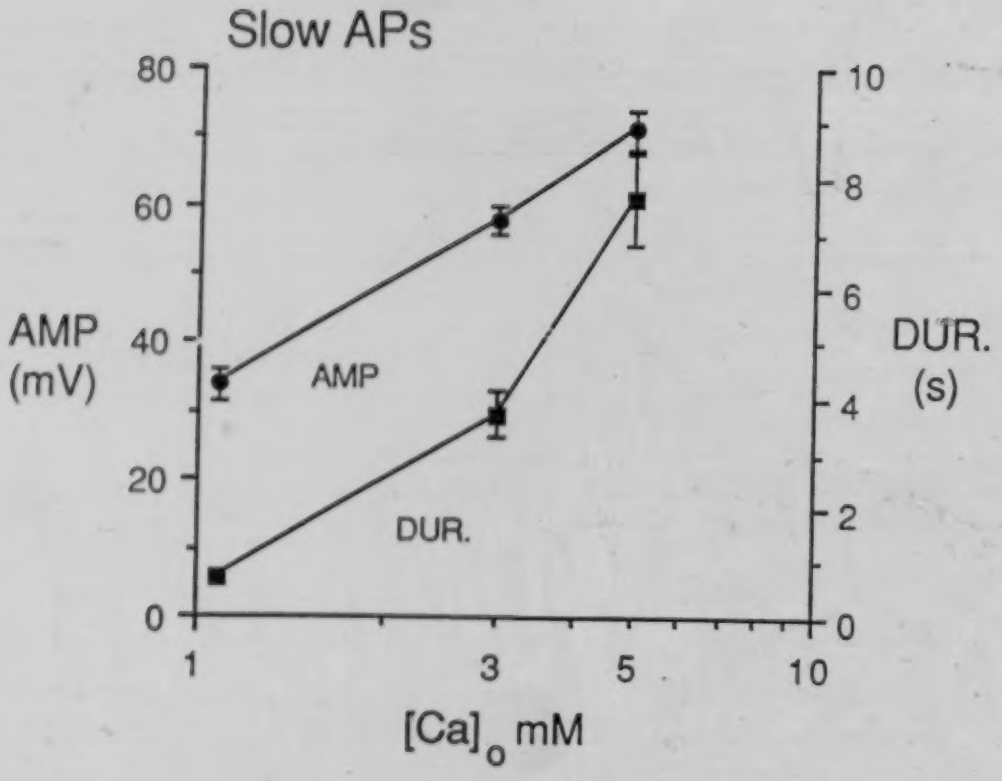

FIG. 12 Amplitude (left ordinate) and duration (right ordinate) as a function of increasing $[Ca]_o$ (mM) plotted on a semilog scale. Symbols represent mean ± S.E. When a standard error is not shown it was less than the size of the symbol. Circles, amplitude (mV); squares, duration (s).

$[Ca]_o$ concentrations. For the precise estimation of the slope, it would be necessary to test more than three $[Ca]_o$ concentrations. Duration (measured at 50% amplitude) of the slow APs increased nonlinearly with the elevation of $[Ca]_o$ (Fig. 12, filled squares). Fig. 13 shows the increase in the area of Ca^{++}-dependent slow APs as a function of $[Ca]_o$. Several findings indicate that these slow APs are produced by Ca^{++} influx through voltage-dependent slow calcium channels:

1) Na^+ cannot be participating in this response, as the solution is Na^+-free and the fast sodium channels have been inactivated by the addition of 20 mM K^+.

2) The slow APs are dependent on $[Ca]_o$, both their amplitude and their duration increases with increasing $[Ca]_o$ (Fig. 12 and Table 7).

3) Ca^{++}-channel blockers such as nifedipine (10^{-5} M, n=3) and diltiazem (10^{-5} and 10^{-7} M, n=3 each) blocked these slow APs (Fig. 14).

4) These slow APs primarily originate in the t-tubules, since they can be abolished by glycerol shock treatment, which dissociates the t-tubules from the surface membrane [192]. The slow Ca^{++} channels are primarily located in the t-tubules of skeletal muscle twitch fibres [118, 142].

4.6.2 Effect of non-peptide opioids on Ca^{++}-dependent slow APs

Typical Ca^+-dependent slow APs obtained using Cl^--free, Na^+-free, high-K^+ solution ($[Ca]_o$=3 mM) and the inhibition of these slow APs produced by the opioid agonists, methadone (10^{-6} M) and U-50,488H (10^{-5} M) are shown in Fig. 15. In a

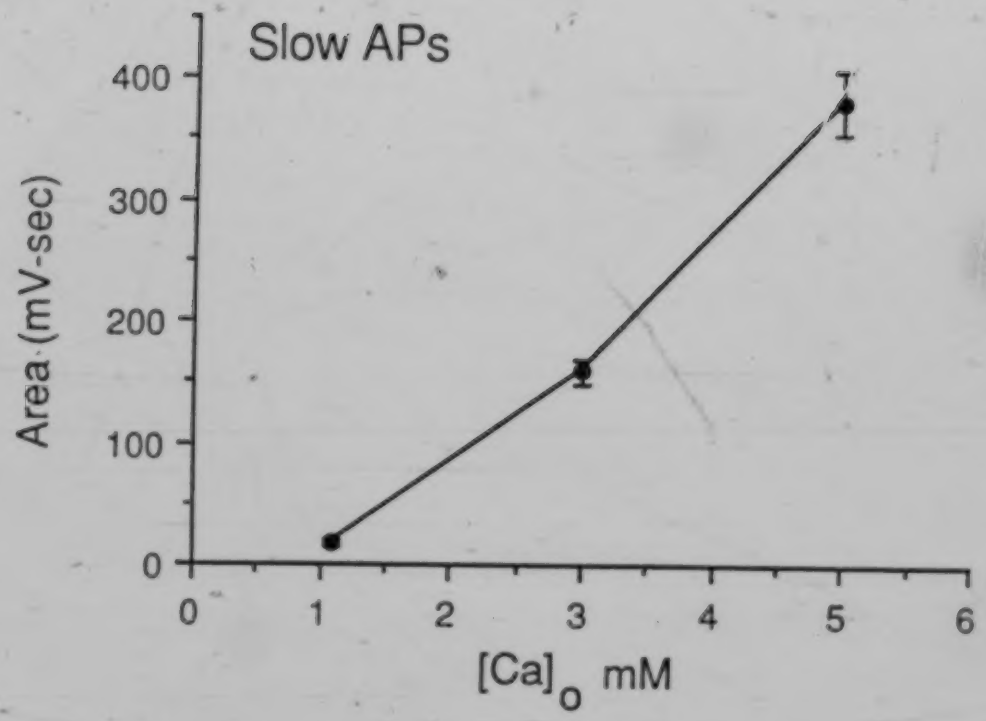

FIG. 13 Area (mV-sec) of Ca^{++}-dependent slow APs as a function of increasing $[Ca]_o$. Values are means ± S.E.M.

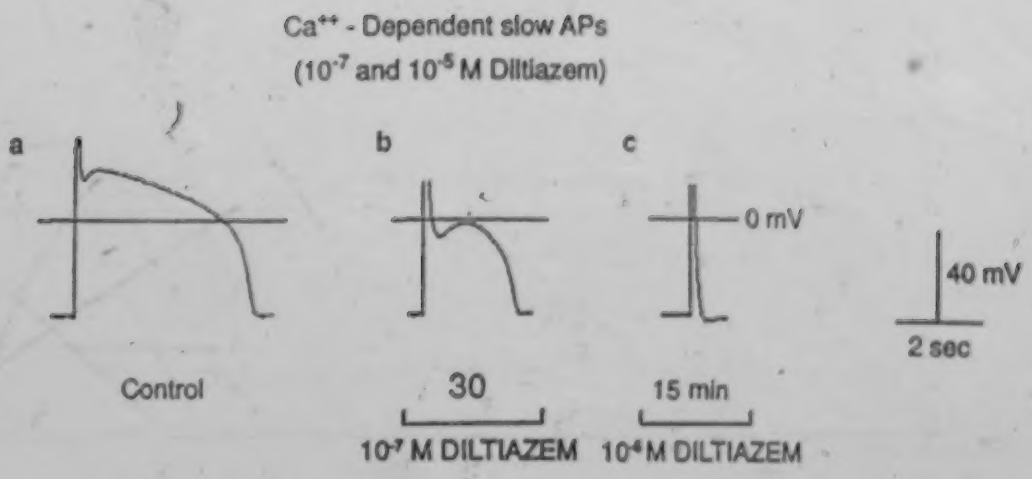

FIG. 14 Effect of diltiazem (10^{-5} and 10^{-7} M) on Ca^{++}-dependent slow APs. Slow APs were obtained using a Na^{+}-free (sucrose substituted), Cl^{-}-free (acetate substituted), high-K^{+} (20 mM) medium. Horizontal line under the records indicates exposure to diltiazem. Times below the records indicate the time after the start of exposure to diltiazem. (a) Control response; (b) test during exposure to 10^{-7} M of diltiazem; (c) test obtained during exposure to diltiazem (10^{-5} M). The upper horizontal line in each panel give the zero potential level. A prominent stimulus artifact is visible at the start of each slow AP.

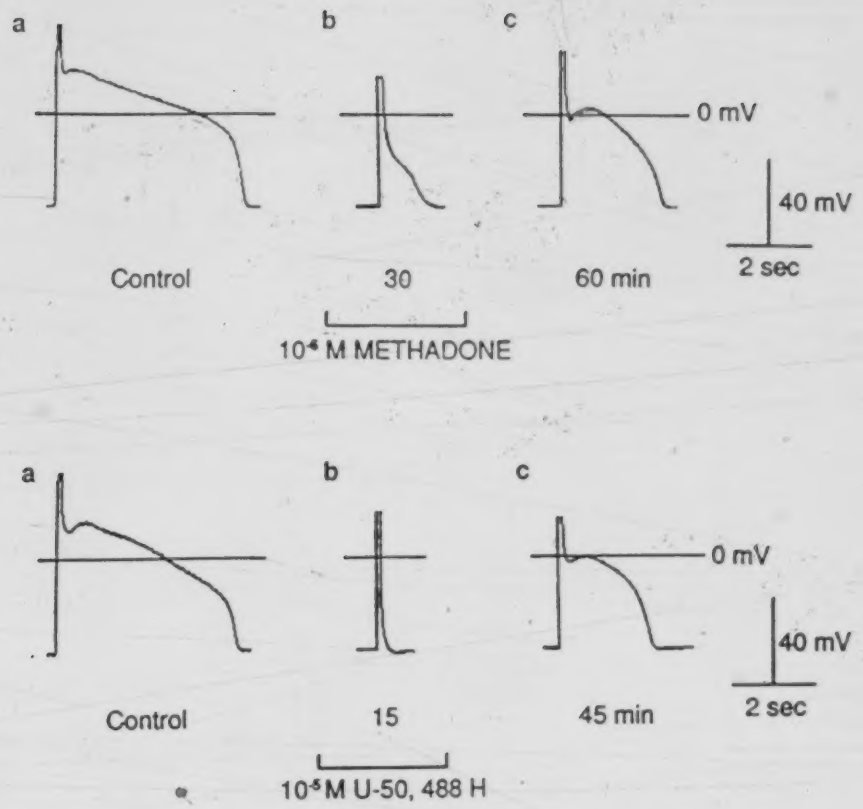

FIG. 15 Effects of methadone (10^{-6} M) and U-50,488H (10^{-5} M) on Ca^{++}-dependent slow APs in frog sartorius muscles. Slow APs were recorded every 15 min. (a) Control response; (b) test during exposure to a drug; (c) recovery response after keeping the muscle for 30 min in a drug-free solution. The upper horizontal line in each panel give the zero potential level. The tops of the stimulus artifacts have been removed.

similar way, Ca^{++}-dependent slow APs obtained by using 3 mM $[Ca]_o$ were inhibited by several other non-peptide opioid agonists and the antagonist, naloxone in a concentration-dependent manner (Table 8).

All the opioids tested produced essentially similar qualitative changes. Thus, it took usually 15 to a maximum of 45 min (in few cases) to develop the maximum effect of the opioids on the Ca^{++}-dependent slow APs, irrespective of the concentration of the opioid applied. Usually, a recovery to 30-70% of control was observed in 1 hr following drug removal. All the non-peptide opioids tested produced a more dramatic decrease in the duration of the slow APs than in the amplitude of the slow APs. In few experiments, tetraethylammonium (TEA) (40 mM) was added to the experimental solution to block K^+ conductance. Meperidine (10^{-5} M) and Methadone (10^{-6} M and 10^{-7} M) caused 82.1, 84.2 and 66.6% inhibition of the area of Ca^{++}-dependent slow APs, respectively (n=3, each) even in the presence of TEA.

4.6.3 Effect of opioid peptides on Ca^{++}-dependent slow APs

Although the non-peptide opioids mentioned above were quite effective in reducing or blocking these APs in frog skeletal muscle, various opioid peptides showed little or no effect on these slow APs (Table 9). Thus, dynorphin (10^{-5} M) caused only a 8.8% inhibition of the area of Ca^{++}-dependent slow APs when tested for 150 min (Fig. 16). Leucine-enkephalin in a concentration as high as 10^{-4} M produced only a 17.9% inhibition of the area of these APs.

TABLE 8

Inhibition of Ca^{++}-dependent slow APs by various non-peptide opioids in frog skeletal muscle

Drug	Maximum Inhibition of Area* (%)			
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Morphine	--	88.7 ± 5.1 (3)	--	--
Meperidine	--	83.5 ± 2.3 (3)	--	--
Methadone	--	98.3 ± 1.7 (3)	80.8 ± 5.5 (3)	68.0 ± 1.0 (2)
Levorphanol	98.3 ± 1.7 (3)	73.7 ± 6.5 (3)	55.8 ± 1.9 (3)	--
Dextrorphan	97.3 ± 1.8 (3)	70.5 ± 3.9 (3)	57.4 ± 1.3 (3)	--
Naloxone	75.0 ± 4.6 (3)	44.6 ± 5.5 (3)	9.1 ± 3.2 (3)	--
U-50,488H	--	100.0 ± 0.0 (3)	81.2 ± 5.0 (3)	43.2 ± 8.3 (3)

NOTE: Mean ± S.E.M.; -- , not tested. Number in bracket = number of experiments.

* Area under the slow AP, eliminating the stimulus artifact, was measured using the Turbo Pascal program.

TABLE 9

Lack of effect of opioid peptides on the Ca^{++}-dependent slow APs in frog skeletal muscle

Drug	Maximum Inhibition of Area* (%)		
	10^{-4} M	10^{-5} M	10^{-8} M
Leu-enkephalin	17.9 ± 4.2 (3)	---	---
Morphiceptin	---	10.7 ± 4.2 (3)	---
Dynorphin	---	8.8 ± 3.8 (3)	3.7 ± 1.8 (3)

NOTE: Means ± S.E.M., ---, not tested.
Number in bracket = number of experiments.
* Area under the slow AP, eliminating the stimulus artifact, was measured using the Turbo Pascal program.

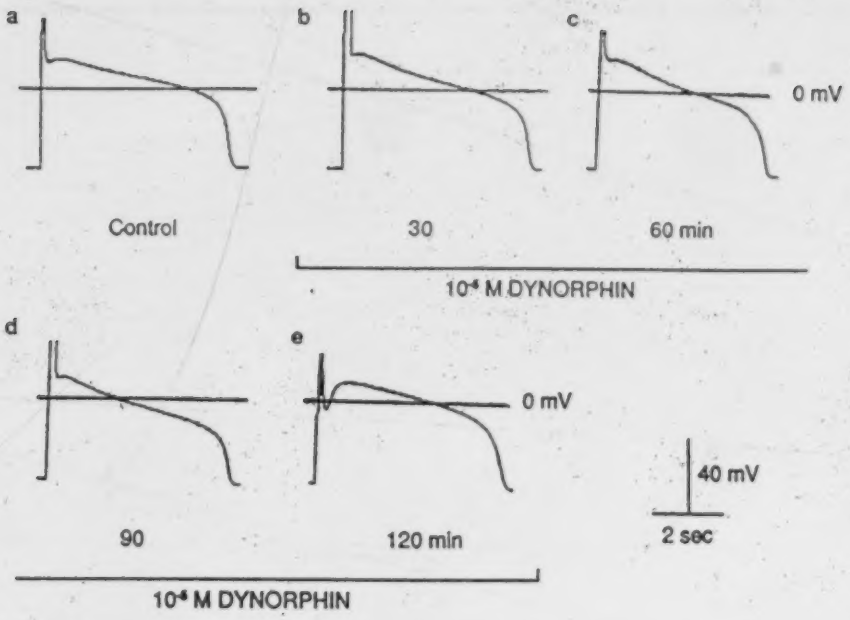

FIG. 16 Lack of effect of dynorphin (10^{-5} M), an opioid peptide, on Ca^{++}-dependent slow APs produced in frog sartorius muscle. Slow APs were obtained using a Na^{+}-free, Cl^{-}-free, high-K^{+} medium and recorded every 15 min. Horizontal line under the records indicates exposure to dynorphin (10^{-5} M). Times below the records indicate the time after the start of exposure to dynorphin. (a) control response; (b) to (e) tests during exposure to dynorphin. The upper horizontal line in each panel give the zero potential level. A prominent stimulus artifact is visible at the start of each slow AP.

4.7 INVESTIGATION OF THE POSSIBLE INVOLVEMENT OF A STEREOSPECIFIC OPIOID RECEPTOR IN THE DEPRESSION OF Ca^{++}-DEPENDENT SLOW APs PRODUCED BY OPIOIDS

4.7.1 Studies with opioid stereoisomers

Levorphanol, a potent opioid analgesic, and dextrorphan, its corresponding inactive isomer, exhibited no significant differences in their ability to produce inhibition of Ca^{++}-dependent slow APs (Fig. 17). This result clearly demonstrated that the effective opioids produced a nonstereospecific blockade of the voltage-dependent slow calcium channels in frog skeletal muscle.

4.7.2 Studies with opioid antagonists

To test this point further, naloxone was used as an opioid antagonist. As with K^+ contractures, when naloxone was tested alone in high concentrations, it produced inhibition of slow APs (Table 8). Since lower concentrations of naloxone (10^{-6} M and lower) showed little effect on Ca^{++}-dependent slow APs, these concentrations were used to test for an antagonistic effect. However, no antagonism was observed with naloxone in concentrations ranging from 10^{-10} M to 10^{-6} M (Table 10). In fact, there seemed to be additive effects.

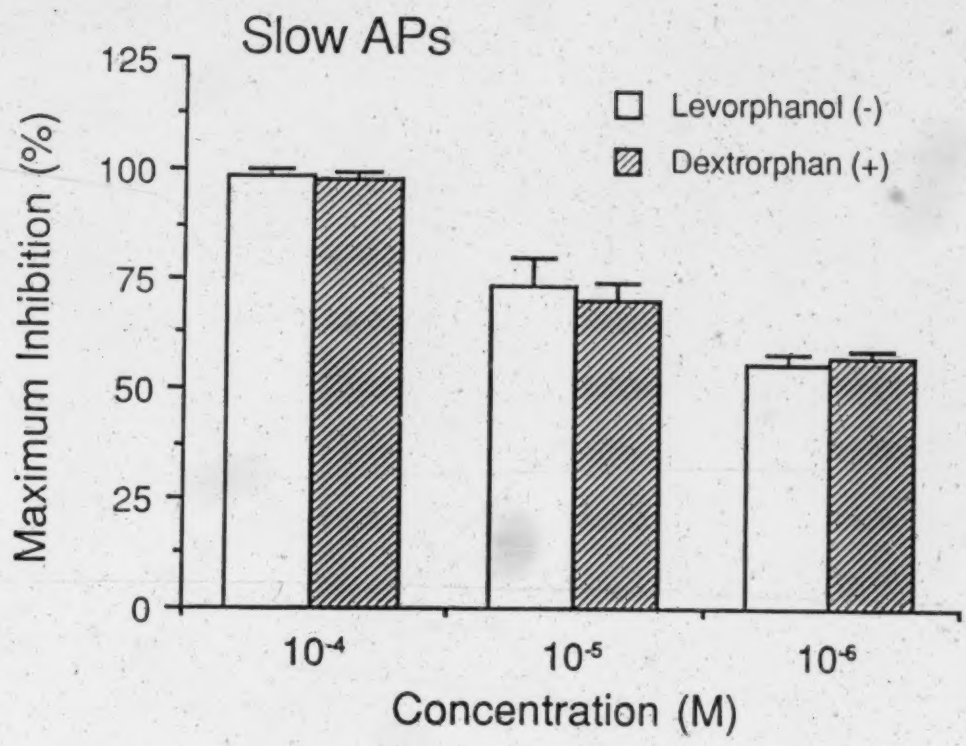

FIG. 17 Maximum inhibition (%) of the area of Ca^{++}-dependent slow APs produced by different concentrations of stereoisomers; levorphanol, analgesically active isomer, and dextrorphan, an inactive isomer, in frog skeletal muscle. Means ± SEM (n=3, each).

TABLE 10

Lack of antagonism of naloxone and norbinaltorphimine on the slow AP inhibition produced by opioid agonists in the isolated sartorius muscle of frog

Agonist (concen)	+	Antagonist (concen)	Maximum inhibition of area (%)	Difference from control value* (%)
Methadone (10^{-6} M)	+	Naloxone (10^{-6} M)	91.0 ± 1.5 (3)	+ 10.2
Methadone (10^{-6} M)	+	Naloxone (10^{-8} M)	86.8 ± 7.7 (3)	+ 6.0
Dextrorphan (10^{-5} M)	+	Naloxone (10^{-10} M)	82.7 ± 4.9 (3)	+ 12.2
Dextrorphan (10^{-5} M)	+	Naloxone (10^{-9} M)	73.3 ± 6.1 (3)	+ 2.8
U-50,488H (10^{-6} M)	+	Naloxone (10^{-7} M)	84.4 ± 4.8 (3)	+ 3.2
U-50,488H (10^{-6} M)	+	Naloxone (10^{-9} M)	84.5 ± 6.0 (3)	+ 3.3
U-50,488H (10^{-6} M)	+	Norbinaltorphimine (10^{-6} M)	83.4 ± 3.1 (3)	+ 2.2
U-50,488H (10^{-6} M)	+	Norbinaltorphimine (10^{-8} M)	85.8 ± 3.1 (3)	+ 4.6

NOTE: Mean ± S.E.M.
Number in bracket = Number of experiments
* See Table 8 for control values (i.e., % inhibition in absence of antagonist)

Naloxone did not antagonize the slow AP inhibition produced by U-50,488H, an opioid agonist specific for the K-receptor subtype (Table 10). Also, norbinaltorphimine, an opioid antagonist specific for the K-receptor subtype showed no antagonism of the inhibition of the slow APs produced by U-50,488H (Table 10). Thus, both naloxone and norbinaltorphimine did not produce any antagonism of the U-50,488H induced depression of Ca^{++}-dependent slow APs.

The results presented above were used to establish a relationship between the inhibitory effects of opioids on K^+ contractures (Table 3 and 4) and Ca^{++}-dependent slow APs (Table 8 and 9). A linear and highly significant correlation (r=0.96) appears to exist between K^+ contracture and Ca^{++}-dependent slow AP inhibition by opioid drugs (Fig. 18). Thus, in general, same rank order of activity was observed despite quantitative differences in apparent potency.

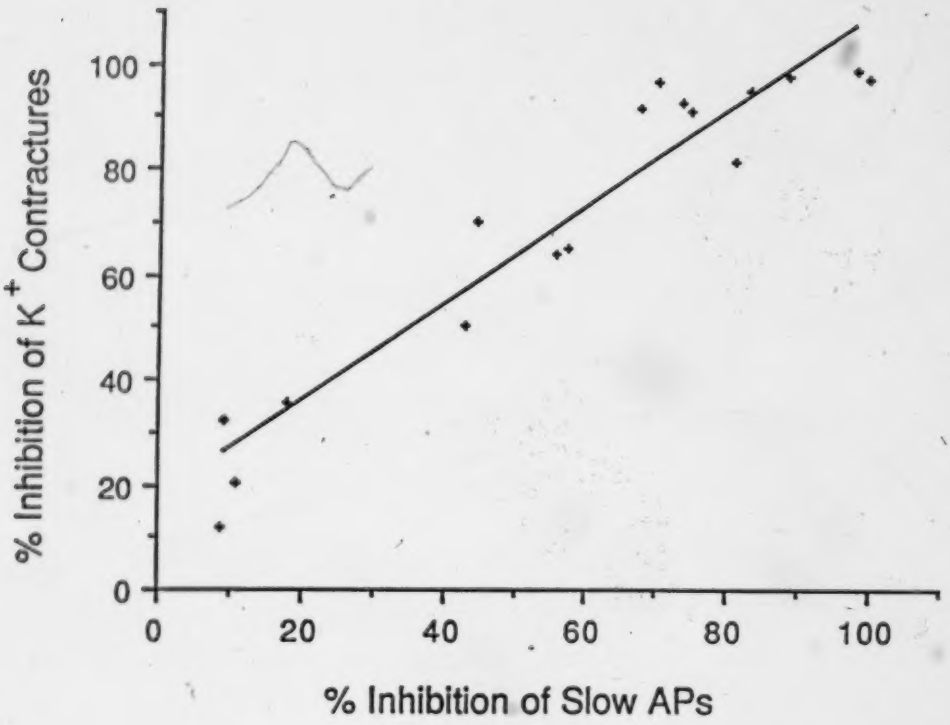

FIG. 18 Correlation between the inhibitory effects of opioids on Ca^{++}-dependent slow APs and K^+ contractures. The plot has a slope of 0.91 and a correlation coefficient r=0.96.

4.8 EFFECT OF FREQUENCY OF K^+ CONTRACTURE TESTS ON THE BLOCK PRODUCED BY NON-PEPTIDE OPIOIDS

As shown earlier, several non-peptide opioids inhibited K^+ contractures (123 mM) in a concentration-dependent manner (Table 3). Little or no reduction of the K^+ contracture was observed in the first test with the high-K^+ solution following the drug application (Fig. 3). However, when tests were repeated at 15 min intervals, block of K^+ contractures was eventually produced (Fig. 3). Thus, it took about 60 min for the complete block to occur with methadone (10^{-5} M), when K^+ contractures were tested every 15 min. This blockade of K^+ contractures by non-peptide opioids was partially reduced when the muscles were kept in the drug solution without testing with the high-K^+ solution for 30 min or more. Thus, methadone (10^{-5} M) completely blocked K^+ contractures in 60 to 90 min when tested with the high-K^+ solution every 15 min. This block was partially recovered (33.5% recovery) by testing the K^+ contracture after a 30 min rest (n=3).

A more complete picture of the effects of methadone (10^{-5} M) on K^+ contractures is presented in Fig. 19. In the experiment shown (Fig. 19), one muscle (filled circles) was tested with the high-K^+ solution every 15 min in methadone (10^{-5} M) and the other muscle (open circles) from the same frog was only tested after 90 min in methadone (10^{-5} M). While in the first toe muscle (filled circles), K^+ contractures were blocked by methadone (10^{-5} M) in 75 min, little or no decrease in

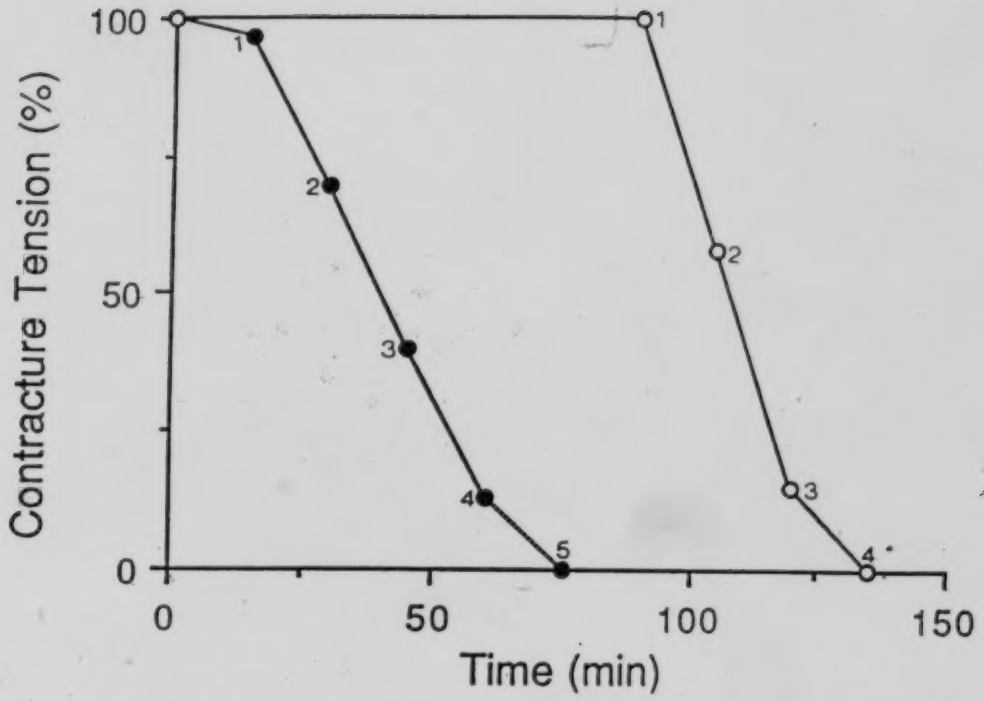

FIG. 19 Effect of timing of K^+ contracture tests on the inhibition produced by methadone (10^{-5} M) in a pair of toe muscles isolated from a single frog. Muscle put in a drug containing solution at time zero. One muscle was tested with high-K^+ solution every 15 min (filled circles, 1-5) and complete inhibition of high-K^+ contracture was observed by the fifth test with the high-K^+ solution. Another muscle from the same frog was tested with high-K^+ solution after a 90 min exposure to methadone (10^{-5} M) (open circles, 1) and no inhibition of high-K^+ contracture was observed. Treating the same muscle further every 15 min produced complete block of K^+ contractures by the fourth test with the high-K^+ solution (open circles, 4).

the initial response of the high-K^+ solution was obtained with the second toe muscle (open circles) after a 90 min exposure to methadone (10^{-5} M) (Fig. 19, n=3). Subsequently, testing the second toe muscle (open circles) every 15 min eventually blocked K^+ contractures (Fig. 19). Similar results were obtained with U-50,488H (10^{-5} M), *i.e.*, a complete block of the K^+ contractures in 60 to 75 min when the K^+ contractures were tested every 15 min and little or no effect on the first K^+ contracture even after a long (90 to 120 min) exposure to U-50,488H (10^{-5} M) (n=3).

Using methadone (10^{-5} M) as a prototype, a more detailed study was conducted of the effect of changing the frequency of K^+ contracture tests; as shown in Fig. 20, the inhibition could be increased (or accelerated) by increasing the frequency of the K^+ contracture tests. Thus, when these contractures were evoked every 10 min, complete block was obtained by 40 min, while it took about 75 min for the complete block to occur when contractures were evoked every 15 min (n=3, each). With high-K^+ tests every 30 min, the same concentration of methadone produced only a 48% inhibition, while this block completely disappeared, if high-K^+ tests were made every 60 min (n=2, each) (Figs. 20 and 21). Thus, blockade of K^+ contractures by non-peptide opioids was found to be dependent on the frequency of K^+ contracture tests.

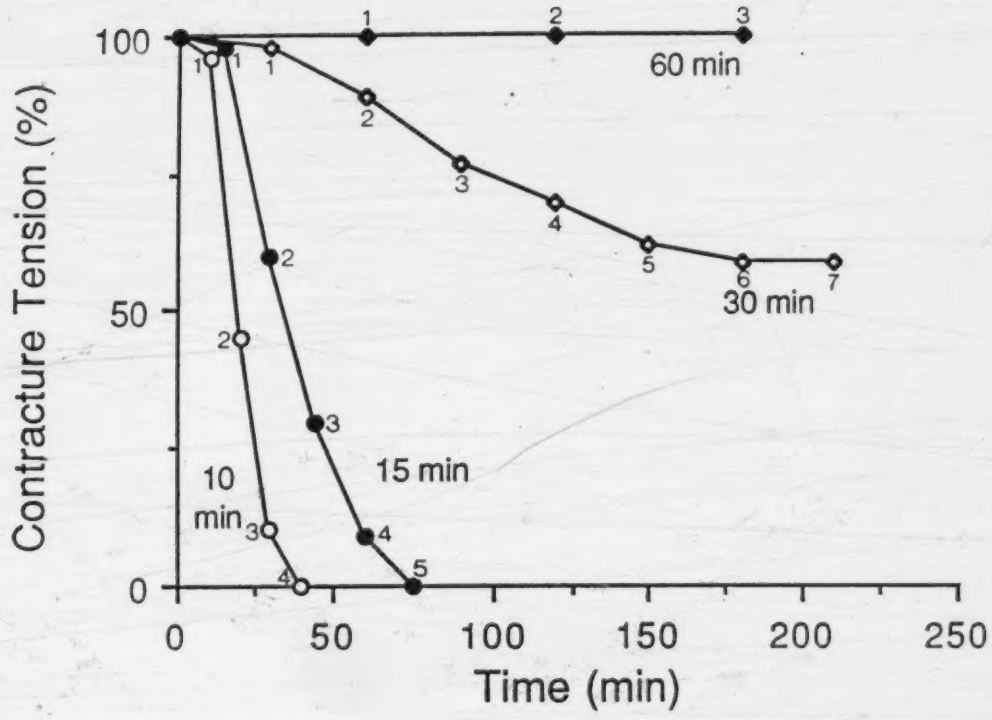

FIG. 20 Inhibition of K^+ contractures produced by methadone (10^{-5} M), dependent on the frequency of K^+ contractures produced in frog toe muscle. Frequency of K^+ contractures: every 10 min (0-0, 1-4), every 15 min (●-●,1-5), every 30 min (◇-◇, 1-7) and every 60 min (◆-◆, 1-3). Numbers indicate the application of high-K^+ solution with different intervals. For details see the text.

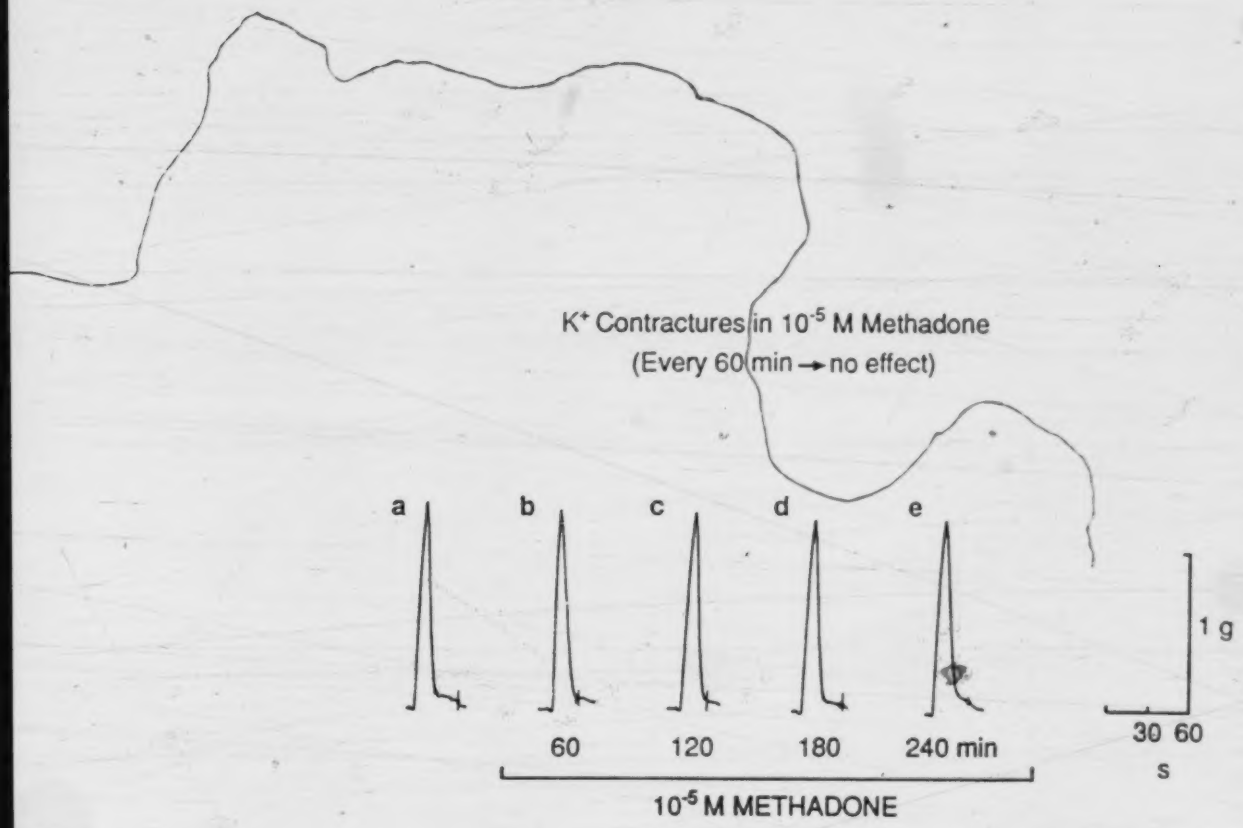

FIG. 21 Lack of effect of methadone (10^{-5} M) when K^+ contractures (123 mM) were induced every 60 min. Horizontal line under the records indicates exposure to methadone (10^{-5} M) in choline-Ringer's solution. Times below records indicates the time after exposure to methadone. (a) Control response; (b - e) tests during exposure to methadone.

4.9 EFFECT OF A Cl^--FREE, HIGH-K^+ SOLUTION ON THE K^+ CONTRACTURE INHIBITION PRODUCED BY NON-PEPTIDE OPIOIDS

The inhibitory effect of the non-peptide opioids on K^+ contractures developed slowly. Even with higher concentrations (10^{-5} M and more), it took a minimum of 60 min for the complete block of K^+ contractures by non-peptide opioids (see, section 4.1.2). In contrast, the blockade of the Ca^{++}-dependent slow APs by the non-peptide opioids was produced in about 15 min, irrespective of the concentration of the drug employed (see, section 4.6.2). To resolve this discrepancy, a Cl^--free, high-K^+ (10 mM) solution was employed. This experimental solution was similar to the solution used for generating Ca^{++}-dependent slow APs. The effect of this particular experimental solution on the non-peptide opioid-induced block of the K^+ contractures was studied.

In a Cl^--free, Na^+-Ringer's solution with normal $[K]_o$ (2.47 mM, RMP= -90 mV), it took an average 75 min (n=3) for the complete block of the K^+ contactures with methadone (10^{-5} M), which is the same time as is required for blocking K^+ contractures using a choline-Ringer's solution. Increasing $[K]_o$ in a Cl^--free solution to 5 mM (RMP=-75 mV) reduced the time to an average of 60 min for the complete block of the K^+ contractures (n=3). A further increase in $[K]_o$ to 10 mM (RMP=-60 mV), resulted in a complete block of the K^+ contractures by the first test (15 min) with high-K^+ solution in methadone (10^{-5} M) (Fig. 22, n=3).

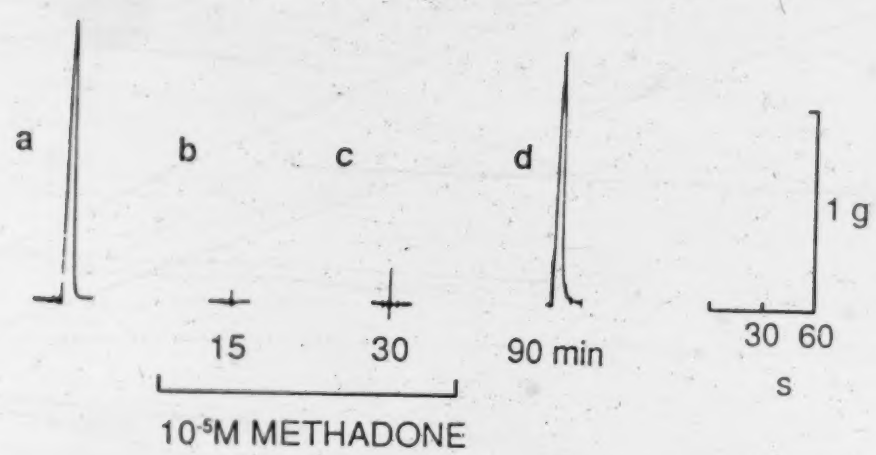

FIG. 22 Blockade of K^+ contracture produced by the first test (15 min) with high-K^+ solution by methadone (10^{-5} M) in a Cl^--free, high-K^+ (10 mM) medium. Times below the records indicates the time after exposure to methadone (10^{-5} M), (a) control response; (b) and (c) tests during exposure to methadone; (d) recovery response after 60 min.

Similarly, U-50,488H (10^{-5} M) completely blocked the K^+ contractures by the first test (15 min) in a Cl^--free, high-K^+ (10 mM) solution (n=3). In separate experiments, U-50,488H (10^{-7} M) produced a 98% inhibition of the K^+ contractures in a Cl^--free, high-K^+ solution, whereas in normal Ringer's solution this concentration produced only a 45% inhibition of the K^+ contractures (n=3, each).

4.10 MISCELLANEOUS PRELIMINARY EXPERIMENTS

4.10.1 Effect of Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) on K^+ contractures and Ca^{++}-dependent slow APs

DTALE has been reported to have calcium-agonist activity in a vascular smooth muscle preparation [193]. This calcium-agonist effect of DTALE was found to be naloxone-insensitive, but it could be reversed by the calcium-channel antagonists verapamil and nitrendipine [193]. Thus, it was of interest to study the effect of DTALE on a skeletal muscle preparation.

The results obtained in one of the experiments with DTALE (10^{-5} M) are shown in Fig. 23. In this experiment, DTALE (10^{-5} M) did not produce any effect on submaximal K^+ contractures (25 mM). Similarly, 10^{-4} M (n=3) and 10^{-5} M (n=3) DTALE had no effect on 25 and 40 mM K^+-induced contractures, when tested for 150 min. In other experiments, DTALE (10^{-5} M, n=3) produced no effect on Ca^{++}-dependent slow APs.

4.10.2 Effect on haloperidol on voltage-dependent slow calcium channels

Recently, haloperidol was reported to bind with high affinity to σ-receptor sites [194,195]. Therefore, it was of interest to study effect of haloperidol on voltage-dependent slow calcium channels in skeletal muscle.

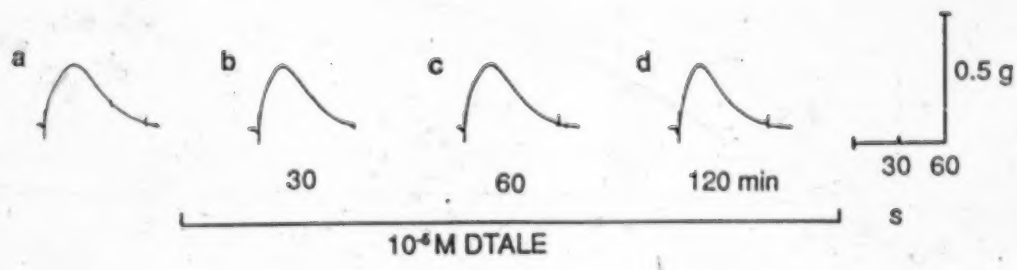

FIG. 23 Lack of effect of Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) on K^+ contractures (25 mM) of frog toe muscle. Tests were made every 15 min. Horizontal line under the records indicates exposure to DTALE. Times below records indicate the time after the start of the exposure to DTALE. (a) Control response; (b - d) tests during exposure to DTALE.

Haloperidol in concentrations of 10^{-5} M and 10^{-6} M produced a 100% (n=3, Fig. 24) and a 56% (n=3) inhibition of K^+ contractures, respectively. Similarly, haloperidol (10^{-5} M, n=3) completely blocked Ca^{++}-dependent slow APs in frog skeletal muscle. In a separate experiement, haloperidol (10^{-5} M) also caused a 70% inhibition of caffeine contractures (n=1). In the same concentrations (10^{-5} M and 10^{-6} M), it also produced a 93% (n=3, Fig. 25) and a 65% (n=3) inhibition of twitches in isolated frog toe muscles. For comparison, effects of methadone on the twitch responses were studied. By contrast to haloperidol, methadone produced a marked twitch potentiating effect. Thus, methadone (10^{-4} M and 10^{-5} M) caused a 175% (n=3, Fig. 25) and a 140% (n=3) potentiation of twitch responses, respectively.

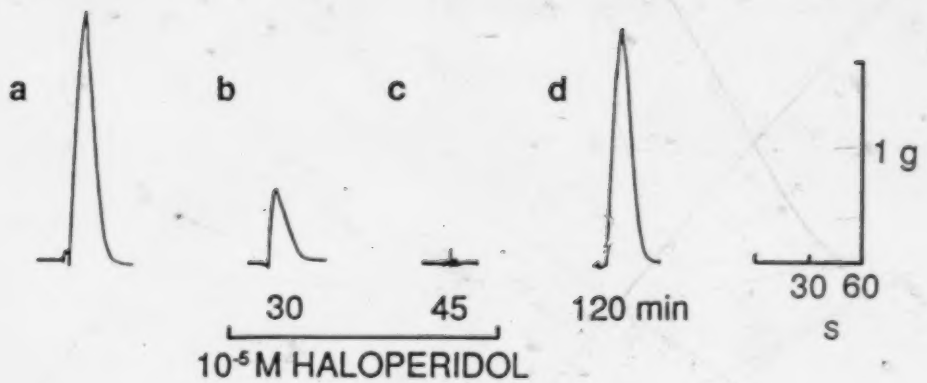

FIG. 24 Effect of haloperidol (10^{-5} M), a σ-receptor agonist, on K^+ contractures (123 mM) of frog toe muscle. Tests were made every 15 min. Horizontal line under the records indicates exposure to haloperidol. Times below records indicate the time after the start of the exposure to haloperidol. (a) Control response; (b - c) tests during exposure to haloperidol; (d) recovery response.

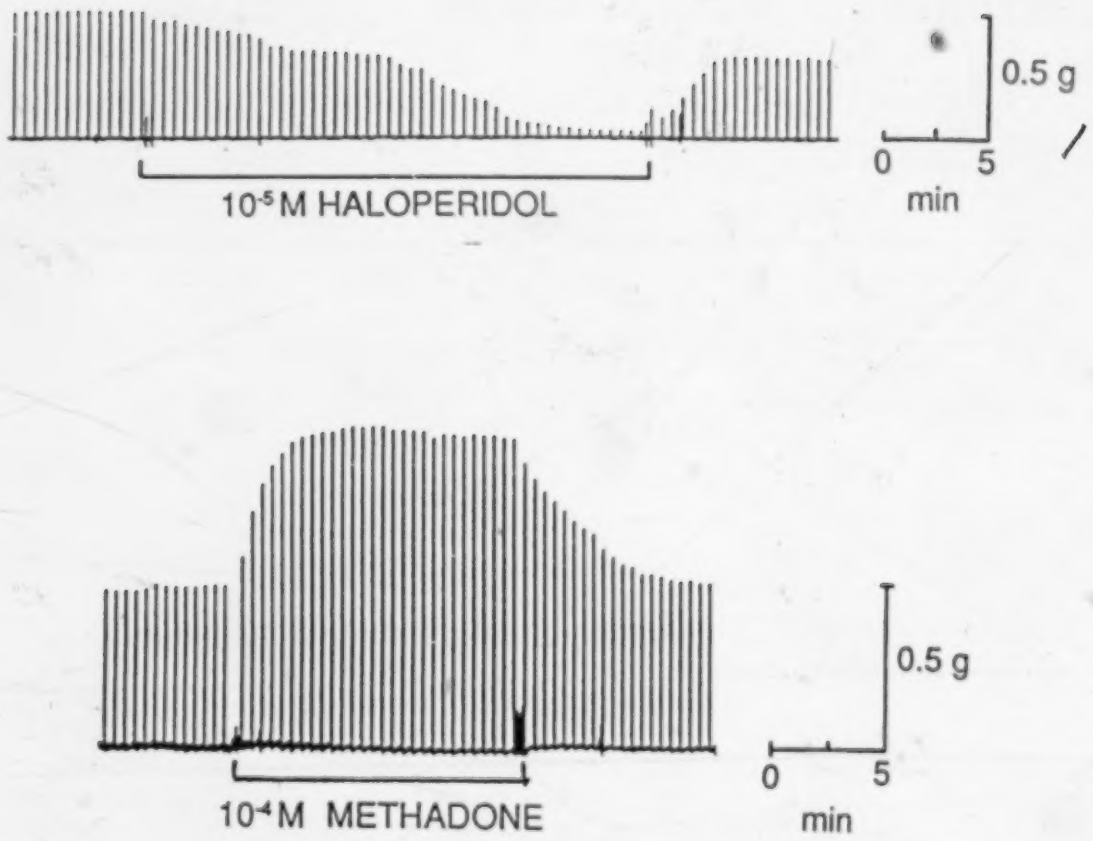

FIG. 25 Twitch inhibition and potentiation in the isolated frog's toe muscle produced by haloperidol (10^{-5} M) and methadone (10^{-4} M), respectively. The preparation was directly stimulated supramaximally once every 30 sec. Horizontal line under the records indicates exposure to a drug.

5. DISCUSSION

5.1 Inhibition of K^+ contractures and Ca^{++}-dependent slow APs by non-peptide opioid drugs

During a study of the ability of many opioid drugs to potentiate twitches in skeletal muscle, a nonstereospecific effect, it was observed that methadone blocked K^+ contractures [180]. To determine whether the inhibition of K^+ contractures caused by methadone was a property unique to that compound, the effects of several opioids with diverse chemical structures were tested on K^+ contractures in frog skeletal muscle. The results of this study were that all of the non-peptide opioids tested produced an inhibition of K^+ contracture similar to that produced by methadone (Table 3). Thus, inhibition of K^+ contractures was observed with different non-peptide opioids, each believed to interact with a particular opioid receptor subtype; e.g., morphine, meperidine and methadone - μ-receptor agonists; U-50,488H - K-receptor agonist; and naloxone - a non-selective opioid antagonist, all produced this effect (Table 3).

The ability of the opioid antagonist naloxone to produce inhibition of K^+ contractures was not surprising because this compound has been demonstrated to be a 'partial agonist' in frog skeletal muscle [196]. Therefore, if used in high concentrations, it showed agonist-like effects. Such effects were also shown by earlier studies of Frank [68] and Frazier *et al.* [197], wherein they observed that the effects of opioid antagonists in high concentrations add on to the effects of opioid agonists.

Surprisingly, while the various non-peptide opioids tested were quite effective in reducing K^+ contractures in low concentrations, various opioid peptides, e.g., leucine-enkephalin - a δ-receptor agonist; morphiceptin - a μ-receptor agonist; and dynorphin - a K-receptor agonist, all had little or no effect on these contractures (Table 4). Even the enzymatically non-degradable peptide analogue D-Ala-leucine-enkephalinamide, had little effect on K^+ contractures (this point will be discussed further in section 5.3 below).

Attempts to associate the non-peptide opioid-induced inhibition of K^+ contractures with one of the proposed opioid receptor subtypes met with failure. The non-peptide opioid-induced inhibition of K^+ contractures in the frog toe muscle was resistant to antagonism by naloxone. The only noticeable effect of naloxone was an increase in the degree of contracture inhibition (Table 5 and 6). Norbinaltorphimine, an opioid antagonist specific for the K-receptor subtype did not antagonize the inhibition of K^+ contractures produced by U-50,488H, a non-peptide opioid agonist specific for the K-receptor subtype (Table 6, Fig. 7).

Analgesic activity, like most of the actions of the opioid drugs, is highly stereospecific, with almost all the activity residing in levo-isomers. If the inhibition of K^+ contracture involved a stereospecific receptor, the levo-isomer should have had higher potency than the corresponding dextro-isomer. However, this was not the

case. Thus, we found that the analgesically active isomer, levorphanol, and its corresponding inactive isomer, dextrorphan, were equally effective in causing inhibition of K^+ contractures (Fig. 6).

The effects on the K^+ contractures were produced at low opioid concentrations (Table 3). Despite this, the effect was not produced via any known stereospecific opioid receptor. The data suggested that the effect on the K^+ contracture is a consequence of the activation of a nonstereospecific, naloxone-resistant opioid receptor. Interestingly, similar nonstereospecific, naloxone-resistant effects of opioids have been reported by others in frog skeletal muscle [198], guinea-pig ileum [199] and rat vas deferens [200].

One treatment that could antagonize the K^+ contracture inhibition produced by the non-peptide opioids was an elevation of $[Ca]_o$ from 1.08 mM to 5 mM (Fig. 8). This antagonism of the drug effect by Ca^{++} may be explained by two possibilities: (1) competition between drug and Ca^{++} ions for a binding site on or near the slow channel, and (2) the increased electrochemical driving force for Ca^{++} influx through the fraction of slow channels not blocked by the drug. The latter mechanism probably operates in all cases.

Non-peptide opioids in concentrations used to block K^+ contractures had no effect on the K^+-induced membrane depolarization (Fig. 10). Also, non-peptide

opioids had no effect on caffeine-induced contractures, indicating that these drugs did not inhibit the release of Ca^{++} from sarcoplasmic reticulum stores. Thus, morphine (10^{-5} M), which produced almost a complete block of the K^+ contractures, did not reduce the maximum tension of the caffeine-induced contracture (Fig. 9). Also, these findings suggest that the internal contractile apparatus is not modified by the non-peptide opioids. Moreover, in a previous study [198], the non-peptide opioids tested were shown to produce twitch potentiation at these low concentrations and caused twitch inhibition only after exposing the muscles to higher concentrations. Thus, exposing toe muscles to 10^{-3} M morphine reduced the twitch by only 50% after 45 min. Thus, the block of K^+ contractures found in this study occurred at concentrations at which the twitch is either potentiated (Fig. 25) or unaffected. This supports the conclusion that the internal contractile apparatus is not depressed at opioid concentrations that block the K^+ contractures. As caffeine-induced contracture were not blocked, and K^+-induced membrane depolarizations were not reduced, and twitches were either potentiated or unaffected at the drug concentrations used, the block of the K^+ contractures cannot be a nonspecific, local anesthetic-like effect.

These results indicate strongly that the effective non-peptide opioids are inhibiting K^+ contractures in skeletal muscle by blocking the voltage-dependent slow calcium channels located in the t-tubules. To further support this conclusion, the effects of opioids on Ca^{++}-dependent slow APs were studied, using intracellular recording techniques.

Recently, electrophysiological studies have shown that opioids block voltage-dependent slow calcium channels in several preparations. Thus, activation of δ-receptors by enkephalins has been shown to reduce calcium conductance in neuroblastoma x glioma hybrid cells [63]. While activation of δ- as well as μ-receptors has been shown to cause inhibition of calcium currents in human neuroblastoma cells [64]. All of these effects were antagonized by naloxone.

Ca^{++}-dependent slow APs in frog skeletal muscle were induced by using a Cl^--free (acetate substituted), Na^+-free (sucrose substituted), high-K^+ (20 mM) solution [192]. These slow APs are produced by the influx of Ca^{++} ions through the voltage-dependent slow calcium channels located in the t-tubules (this point has been discussed in detail, in section 4.6.1). These APs were dependent on $[Ca]_o$ (Fig. 11 and Table 7) and they were abolished by the organic calcium channel blockers nifedipine and diltiazem (Fig. 14).

Several non-peptide opioids inhibited Ca^{++}-dependent slow APs (Table 8 and Fig. 15). The inhibition of the Ca^{++}-dependent slow APs by non-peptide opioids was not prevented by adding tetraethylammonium (TEA), which is known to block membrane K^+ conductance. This suggested that the effective opioids were not blocking the slow APs by increasing the K^+ conductance, but by a direct blockade of the voltage-dependent slow calcium channels.

As was the case for the K^+ contractures, various opioid peptides tested showed little or no effect on these Ca^{++}-dependent slow APs (Table 9). Thus, dynorphin, which has been shown to block slow APs in mouse dorsal root ganglion neurones [54], did not show any effect on Ca^{++}-dependent slow APs in frog skeletal muscle in concentrations as high as 10^{-5} M (Fig. 16).

As observed during the K^+ contracture studies, inhibition of the Ca^{++}-dependent slow APs by the non-peptide opioids was found to be nonstereospecific and resistant to antagonism by naloxone (Fig. 17, Table 10). There was an excellent correlation (r=0.96) between the inhibitory effects of opioids on K^+ contractures and Ca^{++}-dependent slow APs, showing that opioids that are potent (non-peptide opioids) in inhibiting slow APs are also potent in inhibiting K^+ contractures (Fig. 18).

Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) has been reported to have calcium-agonist activity in a vascular smooth muscle preparation [12]. This activity is resistant to antagonism by naloxone, but could be inhibited or reversed by organic calcium channel blockers. This report prompted us to study DTALE's effects on the skeletal muscle preparation. However, DTALE had no effect on voltage-dependent slow calcium channels in frog skeletal muscle.

Recently, haloperidol has been reported to bind with high affinity to the σ-receptor subtype [194, 195]. Therefore, it was of interest to study its effect on the

voltage-dependent slow calcium channels in skeletal muscle. Haloperidol (10^{-5} M) did block K^+ contractures (Fig. 24) and Ca^{++}-dependent slow APs, indicating its ability to block slow calcium channels. However, this effect of haloperidol was not specific to calcium channels. It also inhibited twitches (Fig. 25) and caffeine-induced contractures in the same concentration range. Haloperidol has also been shown to block Na^+ channels in the same concentration range [208]. Therefore, the effects of haloperidol were not specific or limited to calcium channels.

5.2 Frequency- and voltage-dependent block of K^+ contractures by non-peptide opioids

Inhibition of the K^+ contractures by the non-peptide opioids was found to be dependent upon the frequency of K^+ contracture tests. This inhibition could be increased and/or accelerated by increasing the frequency of the K^+ contracture tests (Fig. 20). Thus, methadone (10^{-5} M) completely blocked K^+ contractures in 75 min when the contracture tests were made every 15 min, and this block completely disappeared if high-K^+ tests were made every 60 min (Figs. 20 and 21). These results indicated that more frequent depolarizations increased the non-peptide opioid block of K^+ contractures. This frequency- (use-) dependent inhibition of K^+ contractures by the non-peptide opioids indicated a preferential binding of the non-peptide opioids to their receptors in the open- and/or inactivated-state of the calcium channels (this point will be discussed in detail in section 5.3 below).

One of the differences noted in the studies of K^+ contractures and Ca^{++} -dependent slow APs was that the block of the Ca^{++}-dependent slow APs by the non-peptide opioids developed much quicker than the block of the K^+ contractures. It took usually 15 min to a maximum of 45 min (in few cases) for these drugs to develop their maximum effect on Ca^{++}-dependent slow APs. By contrast, even with high non-peptide opioid concentrations, it took 60 to 90 min to produce a maximum reduction of the K^+ contractures. To resolve the discrepancy between the kinetics of the drug effects on K^+ contractures and Ca^{++}-dependent slow APs, we employed an experimental solution similar to the one used for generating slow APs. Testing the muscles in this solution both accelerated and potentiated the non-peptide opioid-induced inhibition of K^+ contractures. Methadone (10^{-5} M) took a minimum of 60 min to completely block the K^+ contracture in normal Ringer's solution, but it blocked by the first test (15 min) with the high-K^+ solution in a Cl^--free, high-K^+ (10 mM) medium (Fig. 22). Thus, the difference in the onset of block of the voltage-dependent slow calcium channels in the two types of experiments can be eliminated when the solutions bathing the muscles are made similar. These results suggest that the binding of the non-peptide opioid to its receptor is voltage-dependent, with depolarization favoring the binding and thereby accelerating (and potentiating) the block. The receptors in this situation, that is, when the membrane is partially depolarized, are more readily available for the drug molecules to bind.

5.3 Possible mechanisms for the inhibition of voltage-dependent slow calcium channels by non-peptide opioids

The results discussed thus far shed some light on the possible mechanism(s) involved in the blockade of voltage-dependent slow calcium channels by non-peptide opioids (Fig. 26).

Involvement of a G protein (and consequently of a second messenger system) in the responses to opioid-receptor activation has been extensively documented (see section 1.2.4). Thus, the opioid inhibition of adenylate cyclase and consequently cAMP formation has been demonstrated in mammalian brain [75, 76] and the neuroblastoma x glioma hybrid cell line [77, 78]. However, the possible involvement of a second messenger system (Fig. 26, 2) or a coupling of opioid receptor to the G protein, which directly regulates the calcium channel (Fig. 26, 1), in the non-peptide opioid-induced blockade of voltage-dependent slow calcium channels can be ruled out for two reasons: (1) Drugs which produce their response through a G protein (and consequently through a second messenger system) bind to their receptors at the outer surface of the cell membrane; that is, they act from the extracellular site. If in the present study, similar receptors were involved, then various opioid peptides should have been effective in blocking voltage-dependent slow calcium channels, since they can bind to their receptors at the outer surface of the cell membrane and there is no necessity to penetrate the membrane. However, opioid peptides were clearly

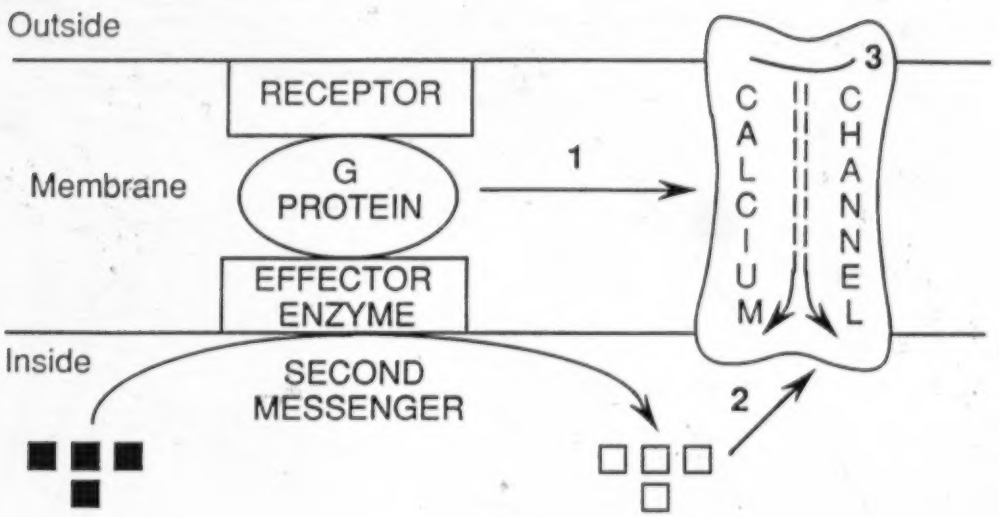

FIG. 26 Schematic representation of possible mechanisms for the non-peptide opioid-induced blockade of voltage-dependent slow calcium channels. (1) Non-peptide opioid receptor coupled to a G protein, which is directly linked to a voltage-dependent slow calcium channel. Thus, activation of opioid receptor will lead to a reduction in calcium current through voltage-dependent slow calcium channels via a G protein that directly regulates the channel activity. (2) Indirect inhibition of slow calcium channels through a 2nd messenger system, e.g., opioid receptor mediated inhibition of adenylate cyclase and consequently cAMP (2nd messenger) formation which will lead to the inhibition of slow calcium channels. (3) Non-peptide opioid binding site inside or near the channel that directly regulates the calcium current through voltage-dependent slow calcium channels.

ineffective in blocking slow calcium channels in skeletal muscle when applied extracellularly. It is possible that the receptor/binding site may be located inside the membrane and since opioid peptides being hydrophilic in nature cannot enter the membrane, and therefore, are ineffective in blocking slow calcium channels. Non-peptide opioids are sufficiently lipophilic to enter/cross the cell membrane, and thus, are effective in blocking the voltage-dependent slow calcium channels in skeletal muscle. However, it is also possible that the receptor is completely different and can be activated only by non-peptide opioids, and not by opioid peptides. (2) In the present study, the non-peptide opioid-induced inhibition of slow calcium channels was found to be frequency- (use-) and voltage-dependent. Such frequency- and voltage-dependent effects are usually not observed with drugs acting through second messenger systems. For example, opioid peptides enkephalin and morphiceptin acting through δ- and μ-receptors, respectively, and involving G proteins in their responses do not show such frequency- or voltage-dependent effects [64].

Although the two reasons presented above make a possible involvement of a G protein (and consequently the second messenger system) seem unlikely, it would be necessary to pursue this possibility further in order to completely rule out the involvement of a second messenger system. In particular, it would be reasonable to test for pertussis toxin-sensitive G protein because most of the responses to opioid-receptor activation studied so far, have shown the involvement of a pertussis toxin-sensitive G protein.

The frequency- and voltage-dependent interaction between the non-peptide opioids and calcium channels in skeletal muscle closely resembles that which occurs between calcium-channel antagonists and calcium channels, and also between local anesthetics and sodium channels [210 - 212]. A simple plugging of the channel by the non-peptide opioids is not consistent with their frequency- and voltage-dependent effects. Our results can be best interpreted within the framework of the modulated receptor hypothesis proposed by Hille [212] to explain the local anesthetic block of sodium channels in nerves and skeletal muscles. In this interpretation, binding of a drug to a receptor located within (Fig. 26, 3) the channel is influenced by the state of the channel which in turn is determined by the membrane potential. This model predicts that ionized drugs can only gain access to the channel-associated receptor via a hydrophilic pathway which is available only when the channels are in the open-state configuration. Uncharged drugs (neutral drugs) can reach the same receptor via this pathway, as well as by a hydrophobic route through the lipid membrane surrounding the channel.

The inhibition of K^+ contractures by non-peptide opioids can be considerably modulated by a change in membrane potential from -90 mV (RMP) to -60 mV. Thus, the inhibition of K^+ contracture by the first test with a high-K^+ solution is observed when the membrane potential is -60 mV and there is no necessity of repeated tests (frequency-) with the high-K^+ solution (Fig. 22). This voltage is too negative to completely open the calcium channels. One interesting possibility that is consistent with the modulated receptor hypothesis is that effective non-peptide

opioids are shifting the steady-state inactivation curve for slow calcium channels towards more negative potentials, that is, in the hyperpolarizing direction. Such a shift in inactivation curve has been often reported with dihydropyridine-type organic calcium-channel blockers [126, 202-204]. Such an effect would suggest that the non-peptide opioids preferentially block channels that are in the inactivated state. However, this possibility remains to be tested.

Another possibility is that the calcium channels are partly but continuously active in partially depolarized muscle. At low levels of K^+ depolarization, there is a sustained influx of calcium in the frog skeletal muscle [205, 206, 207]. Thus, these active calcium channels in partially depolarized muscle might allow the non-peptide opioids to reach their receptors in the channels more effectively than in the normally polarized cells. This would result in quicker block of calcium channels. For this mechanism to occur, drug molecules would have to use a hydrophilic pathway from the cytosolic side of the cell to reach their receptors. The Ca^{++} channel has a selectivity filter of about 6 A° on its extracellular side [214]; this might not allow the non-peptide opioid molecules to enter the channel from the extracellular side, as their molecular size is usually more than 12 A° [216]. So the drugs would have to cross the membrane and enter the channel from cytosolic side in the ionized form. It is interesting to note that most of the non-peptide opioids get extensively ionized at the cytosolic pH and probably enter the channel easily through the hydrophilic pathway. It is also possible that the non-peptide opioid drug molecules may be using

both pathways, that is, hydrophobic pathway through the lipid membrane in their uncharged form and the hydrophilic pathway for the drug molecules, which have crossed the membrane and are ionized in the cytosol. However, more experiments would be necessary to confirm these suggestions. In particular, experiments using the permanently charged (quaternary) non-peptide opioids would be quite useful in testing above possibilities.

5.4 Future recommendations to pursue this project

The voltage-dependent slow calcium channel is a target for a number of drugs collectively called the organic calcium channel blockers. The phenylalkylamines, the benzothiazepines and the 1,4-dihydropyridines, represented by verapamil, diltiazem and nifedipine, respectively, are the best known groups of these compounds. Although these three classes of drugs act at three discrete binding sites on the voltage-dependent slow calcium channels, it has been reported that some new classes of drugs modulate the channel activity by acting at other sites [215]. The L-type calcium channel is a large multimeric protein with five subunits [147]. It is likely that such a large complex will have many drug binding sites. The frequency- and voltage-dependent inhibitory effects of non-peptide opioids on voltage-dependent slow calcium channels are very similar to the effects produced by the organic calcium channel blockers on the voltage-dependent slow calcium channels. Thus, it would be interesting to see if these non-peptide opioids have direct or allosteric effects on the

organic calcium channel blockers' binding sites in skeletal muscle. Whether these opioids define a new site of action remains to be established (Fig. 27).

The effects of non-peptide opioids on the voltage-dependent slow calcium channels in frog skeletal muscle, presented in this study, were nonstereospecific and resistant to antagonism by naloxone. This indicated that a different type of 'opioid drug receptor' was involved. It would be important to determine whether the non-peptide opioids produce similar effects on mammalian skeletal muscles.

Loperamide, an opioid chemically related to meperidine, is clinically used as an anti-diarrhoeal agent. It has a calcium channel blocking effect in taenia coli muscle, which is only partially sensitive to antagonism by naloxone [217, 218]. This inhibitory effect of loperamide on calcium channels has been proposed to contribute to its anti-diarrhoeal effect [218]. It would be interesting to see whether loperamide produces a similar effect on calcium channels in skeletal muscle, and if so, to study the possible involvement of a nonstereospecific, naloxone-resistant receptor in such a response.

Non-opioid antitussive agents, such as dextromethorphan, noscapine, caramiphen have been reported to act through a receptor which is nonstereospecific and naloxone-resistant [1]. In addition, dextromethorphan has been reported to block voltage-dependent slow calcium channels in brain synaptosomes and cultured neural

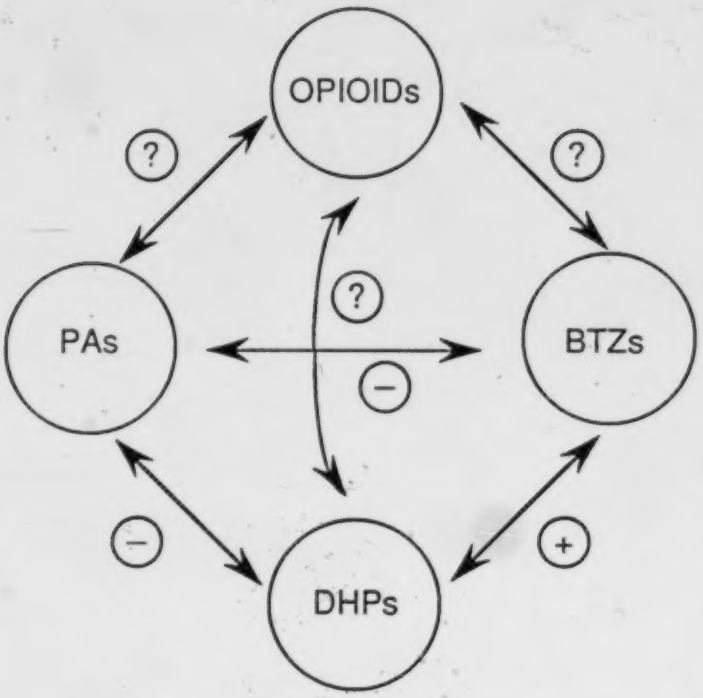

FIG. 27 Schematic arrangement of voltage-dependent slow calcium channel binding sites. The estabalished primary binding sites for 1,4 - dihydropyridines (DHPs), phenylalkylamines (PAs) and benzothiazepines (BTZs) are linked through a series of positive and negative heterotropic interactions (+ and -). Because the identity of the binding site of non-peptide opioids has not been established nor has the heterotropic relationship to other sites been established, this site has been depicted as independent.
(Note: Above figure was modified from ref. [215].)

(PC12) cells [213]. In some preliminary studies from our laboratory, we observed that dextromethorphan did block K^+ contractures in frog skeletal muscle. The inhibitory effects of dextromethorphan on K^+ contractures were observed in concentrations at which the twitch was potentiated. These effects are similar to the inhibitory effects of the non-peptide opioids on voltage-dependent slow calcium channels in skeletal muscle. Thus, it would be important to determine whether dextromethorphan and other antitussive agents produce such an effect through the nonstereospecific, naloxone-resistant receptor involved in the non-peptide opioid-induced blockade of voltage-dependent slow calcium channels in frog skeletal muscle.

6. SUMMARY AND CONCLUSIONS

1. The effects of several opioid drugs were tested on the K^+ contractures of the frog toe muscles. These contractures are produced by the influx of extracellular calcium ions via the voltage-dependent slow calcium channels located in the t-tubules. Methadone and several other non-peptide opioids inhibited K^+ contractures in low concentrations. By contrast, various opioid peptides had little or no effect on these contractures.

2. The inhibition of K^+ contractures by non-peptide opioids was found to be nonstereospecific and resistant to antagonism by naloxone. This implied that the effects of non-peptide opioids are not mediated by any of the proposed opioid receptor subtypes, but are due to activation of nonstereospecific, naloxone-resistant opioid receptors.

3. The inhibitory effects of non-peptide opioids on the K^+ contractures were reversed by raising $[Ca]_o$ from 1.08 mM to 5 mM. This reversal may reflect competition for binding and/or an increase in electrochemical driving force for Ca^{++} influx through the unblocked slow calcium channels.

4. Non-peptide opioids, in concentrations used to block K^+ contractures had no effect on the K^+-induced membrane depolarization. These results indicated that the blocking effects of non-peptide opioids on K^+ contractures were not due to the inhibition of K^+-induced membrane depolarization.

5. These effective non-peptide opioids, in concentrations used to block K^+ contractures had no effect on caffeine contractures. These results indicate that non-peptide opioids neither deplete intracellular calcium stores nor inhibit the release of calcium from intracellular sarcoplasmic reticulum stores. This also indicates that the function of the internal contractile apparatus is not modified by the non-peptide opioids. Moreover, the non-peptide opioids tested were shown in a previous study to produce twitch potentiation or no effect on twitches, in the concentration range used to inhibit K^+ contractures.

6. These effective non-peptide opioids also inhibited Ca^{++}-dependent slow APs in frog skeletal muscle. These slow APs are produced by the influx of calcium ions through the voltage-dependent slow calcium channels. Inhibition of the Ca^{++}-dependent slow APs by the non-peptide opioids was found to be nonstereospecific and resistant to antagonism by naloxone. As was the case with the K^+ contractures, various opioid peptides produced little or no effect on these Ca^{++}-dependent slow APs.

7. The inhibitory effects of non-peptide opioids on the K^+ contractures were found to be frequency- and voltage-dependent. This inhibition could be accelerated (and also potentiated) by increasing the frequency of K^+ contracture tests, and by partial depolarization of the skeletal muscle

membrane in a chloride-free medium. These frequency- and voltage-dependent inhibitory effects are similar to the action of local anesthetics on the sodium channels, and can be interpreted within the framework of the modulated receptor hypothesis. These results indicate a preferential binding of the non-peptide opioids to their receptors in the open- and/or inactivated-state of the calcium channels. It is possible that the non-peptide opioids are shifting the steady-state inactivation curve towards more negative potentials. Such an effect would suggest that the non-peptide opioids preferentially block channels that are in the inactivated state. Another possible explanation is that the non-peptide opioids can reach their receptors inside the channels only when the channels are open. Calcium channels are continuously open in partially depolarized muscle and thus allow the effective opioids to reach their receptors in the channels more effectively than in the normally polarized cells.

8. In a vascular smooth muscle preparation, Des-Tyr-D-Ala-leucine-enkephalinamide (DTALE) has been reported to have calcium-agonist activity, that is resistant to antagonism by naloxone. However, DTALE had no effect on voltage-dependent slow calcium channels in frog skeletal muscle.

9. Haloperidol, which binds to σ-receptors with high affinity, inhibited voltage-dependent slow calcium channels in frog skeletal muscle. However, its effects were not specific to calcium channels. Also it inhibited twitches and

caffeine-induced contractures in the same concentration range. Haloperidol has also been reported to block sodium channels in the same concentration range.

10. It was concluded that several non-peptide opioids block the voltage-dependent slow calcium channels in frog skeletal muscle, thereby blocking K^+ contractures and Ca^{++}-dependent slow APs. This effect was nonstereospecific and naloxone-resistant. By contrast, various opioid peptides showed little or no effect on these channels. These results suggest that there are important differences between the effects of non-peptide opioids and natural endogenous ligands for opioid receptors.

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